Universidad Autónoma de Madrid
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Molecular mechanisms of centromere inheritance in *Xenopus* egg extract

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"If we knew what it was we were doing, it would not be called research, would it?"

Albert Einstein

Mami i tati

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Abstract

Abstract

The centromere is a specialized chromosomal region that provides the foundation for the assembly of the kinetochore, a macromolecular assembly that orchestrates chromosome segregation. In most organisms, centromeres are defined epigenetically by the presence of a histone H3 variant called Centromere Protein A (CENP-A). A group of sixteen proteins that co-immunoprecipitate with CENP-A and localize to centromeres, the Constitutive Centromere Associated Network or CCAN, participate in kinetochore assembly and function. Among them, CENP-C, CENP-T and CENP-W appear to be closest to CENP-A. The propagation and maintenance of this epigenetic mark from one generation to the next is essential for cell division. In this thesis we have used the *Xenopus laevis* egg cell-free system to explore these processes in molecular detail. Chromosomes assembled *in vitro* in these extracts build functional kinetochores and recapitulate most aspects of the CENP-A deposition process described for human cells. Using a combination of biochemistry and fluorescence microscopy we have dissected the contributions of CCAN proteins CENP-C, CENP-T and CENP-W to CENP-A deposition and kinetochore assembly. We found that CENP-C, CENP-T and CENP-W are recruited to centromeres at different times although the three of them are essential for full kinetochore assembly in mitosis. We also established that CENP-C is required for CENP-A loading whereas CENP-T and CENP-W are not. Biochemical characterization of these proteins in the soluble egg extract revealed known and novel interactions among the three proteins and with additional factors. In particular, CENP-C associates with CENP-W and at least three other proteins, HJURP, the CENP-A chaperone; M18BP1, a member of the Mis18 complex, and the chromatin remodeler FACT. While HJURP and M18BP1 had been previously shown to be essential for CENP-A deposition, we demonstrate here that FACT can be detected at centromeres in mitosis and is required for the loading of new CENP-A. We speculate that CENP-C is stored in the egg extract forming a macromolecular complex with many of the proteins implicated in CENP-A assembly in order to facilitate their recruitment to centromeres in the rapid embryonic cycles that follow fertilization.

Resumen

Resumen

El centrómero es el locus cromosómico en el que se construye el cinetocoro, una estructura multiproteica que media la interacción entre los cromosomas y los microtúbulos del huso y que orquesta la segregación cromosómica. En la mayor parte de los organismos, los centrómeros están definidos epigenéticamente por la presencia de una variante de la histona H3 que se denomina CENP-A (de *Centromere Protein A*). Se ha descrito un grupo de dieciséis proteínas centroméricas que interaccionan con CENP-A, que reciben el nombre de CCAN (por *Constitutive Centromere Associated Network*), que son importantes para el correcto ensamblaje y funcionamiento del cinetocoro. Dentro de este grupo, CENP-C, CENP-T y CENP-W parecen estar más próximas a CENP-A. El mantenimiento de la marca epigenética del centrómero de una generación a la siguiente es crucial para una correcta segregación cromosómica. Con el objetivo de entender los mecanismos que regulan la incorporación de CENP-A a la cromatina centromérica y la formación del cinetocoro hemos empleado un sistema *in vitro* basado en extractos obtenidos de huevos de *Xenopus laevis* en los que es posible ensamblar cromosomas con cinetocoros funcionales. Mediante técnicas bioquímicas y microscopía de fluorescencia, en este trabajo nos hemos preguntado acerca de la interacción entre CENP-C, CENP-T y CENP-W, su dinámica de asociación a cromatina, su contribución a la formación del cinetocoro y a la incorporación de nuevos nucleosomas CENP-A que ocurre en cada ciclo celular. Hemos observado que estas tres proteínas se localizan en el centrómero de los cromosomas obtenidos *in vitro* en distintos momentos del ciclo celular si bien las tres son esenciales para la formación del cinetocoro en mitosis. En tanto que CENP-C se requiere para la incorporación de CENP-A, no es ese el caso de CENP-T y CENP-W. Curiosamente, CENP-C aparece en el extracto soluble asociada a HJURP, M18BP1 y el complejo remodelador de cromatina FACT. Estudios anteriores habían descrito el papel de HJURP y M18BP1 en la incorporación de CENP-A. Aquí demostramos por primera vez que FACT juega un papel fundamental en dicha incorporación, al menos en nuestro sistema experimental. Así pues, es posible que CENP-C se almacene en el citoplasma de los oocitos asociado a los principales factores encargados del ensamblaje de cromatina centromérica para guiarlos hacia el centrómero y facilitar este proceso en los primeros ciclos celulares que tienen lugar tras la fertilización.

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Abbreviations

1. Abbreviations

AFM	atomic force microscopy
APC/C	anaphase-promoting complex/cyclosome
bp	base pairs
BSA	bovine serum albumin
CAL1	chromosome alignment defect 1
CATD	CENP-A targeting domain
CBD	CENP-A binding domain
CCAN	constitutive centromere associated network
CDK	cyclin-dependent kinase
CENP-A	centromere protein A
CENP	centromere protein
CHD1	chromodomain helicase DNA binding protein 1
CID	centromere identifier
CSF	cytostatic factor
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DUF	DNA unwinding factor
EDTA	ethylendiaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FACT	facilitates chromatin transcription
h	hours
H3K9me3	histone H3 methylated in lysine 9
H4 Ac	histone H4 acetylated
HAC	human artificial chromosome
HAT	histone acetyltransferase
HDAC	histone deacetylase
HFD	histone-fold domain
HJURP	holliday junction recognizing protein
HSS	high speed supernatant
IgG	immunoglobulin G
INCENP	inner centromere protein

IP	immunoprecipitation
Kb	kilobase
KDa	kilo Dalton
KMN	KNL-1/Mis12 complex/Ndc80 complex
LSD	lysine(K)-specific demethylase
LSS	low speed supernatant
min	minutes
mbp	mega base pairs
RbAp 46/48	retinoblastoma protein (Rb) associated protein 46/48
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
RSF	remodeling and splicing factor
SAC	spindle assembly checkpoint
s	seconds
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interference ribonucleic acid
SMC	structural maintenance of chromosomes
SPT16	suppressor of Ty protein 16
SSRP1	structure-specific recognition protein 1
TBS	Tris Buffered Saline
WB	western blot

Introduction

2. Introduction

Cell division is an essential process by which cells produce its identical offspring. Equal distribution of genetic material to two daughter cells relies on proper segregation of replicated chromosomes. Errors in chromosome segregation can lead to missegregation and, consequently, to changes in chromosome number, a condition which is called aneuploidy. Aneuploidy is the cause of a number of genetic diseases and has also been shown to promote tumorigenesis (Torres et al, 2008; Holland et al, 2009).

Chromosome segregation is directed by a large multi-protein structure called the kinetochore. The kinetochore forms an interface between chromosomes and the microtubules of the mitotic spindle. It provides a site for microtubule attachment and couples spindle forces to move chromosomes during anaphase (Cheeseman and Desai, 2008; Przewloka and Glover, 2009; Takeuchi and Fukagawa, 2012). The kinetochore also mediates the spindle assembly checkpoint (SAC), a signaling pathway that is activated on unattached kinetochores and delays progression into anaphase until all sister chromosomes are correctly attached to opposite poles of the mitotic spindle (Musacchio and Salmon, 2007). Once sister chromatids are separated upon SAC silencing, they can never re-establish the cohesion to repeat the chromosome segregation process. Thus, SAC silencing is a critical decision-making step, which must be triggered only after all chromosomes accomplish bi-oriented attachment (Kops and Shah, 2012). In this way kinetochore ensures that each daughter cell receives a precise complement of the genome.

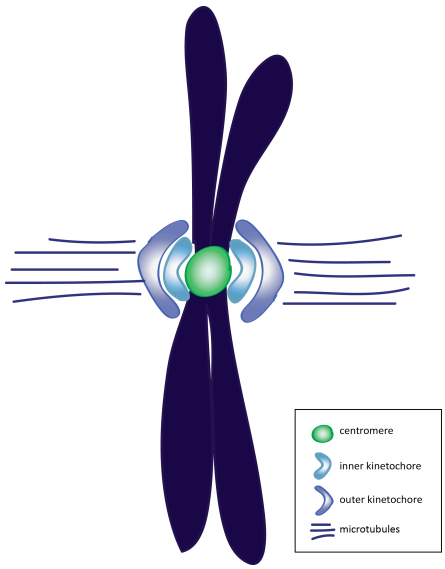


Figure I1. Model of kinetochore structure.
Inner kinetochore proteins assemble upon the centromere in mitosis and provide the platform for the recruitment of the outer kinetochore proteins, which in turn connect to microtubules.

2.1 Centromeres

2.1.1 Sequence composition of the centromere locus

Kinetochores assemble at a single site on each chromosome known as the centromere (Figure I1). Although it provides an essential role that is conserved in all eukaryotes, centromeres are highly variable in sequence and organization between the different species (Figure I2). The *point centromeres* of all 16 chromosomes of *Saccharomyces cerevisiae* are identical and consist of a 125-bp sequence that contains three centromere DNA elements, CDEI, II and III. This DNA is wrapped around a single centromeric nucleosome and attaches to a single microtubule (Herrera et al., 2012; Furuyama and Biggins, 2007). If only a single mutation occurs in one of the DNA elements, it can lead to the loss of centromere function and cell death (Maddox et al, 2012; Cottarel et al, 1989; Spencer and Hieter, 1998). On the other hand, *regional centromeres* are measured in kilobases and do not contain specific sequences but rather repetitive elements. Already in the fission yeast *Schizosaccharomyces pombe*, centromeres consist of 40 to 100 kb of repeated and inverted sequences (Clarke et al, 1986; Fishel et al, 1988). These regions are essential for the establishment and maintenance of the centromeric function, although its sequences are different. The centromeres of *Drosophila melanogaster* have transposon and satellite sequence repeats that can measure up to 420 kb (Murphy and Karpen, 1995; Sun et al., 1997). In humans, centromeres are embedded in the AT-rich alphoid satellite DNA. The 171-bp long repeats are arranged into higher order arrays sizing from 0.2 to 5 Mb (Ugarkovic et al., 2009, Herrera et al, 2012). The α-satellite monomers contain 17-bp long motif known as CENP-B box that is recognized by centromere protein B (CENP-B), which is the only protein that assembles to specific centromeric chromatin sequence (Earnshaw et al., 1987).

2.1.2 CENP-A is the epigenetic mark of the centromere

Except in *S. cerevisiae*, centromere formation does not depend on DNA sequence. The only evolutionary conserved feature of centromeres is that they contain a unique nucleosome in which the canonical histone H3 is replaced by a histone H3 variant called centromere protein A or CENP-A. Due to different moments of their discovery and different naming systems, in some species CENP-A is known under different names. Therefore, in *S. cerevisiae* CENP-A is called Cse4, in *S. pombe* Cnp1, in *D. melanogaster* CID, in *C. elegans* CenH3 (Cleveland et al, 2013). CENP-A is, therefore, the epigenetic mark of the centromere. Reduction or inactivation of CENP-A in different experimental systems causes severe defects in chromosome segregation in all eukaryotes (Allshire and Karpen, 2008). Furthermore,

CENP-A is needed for proper localization of most centromere and kinetochore proteins, while depletion of most of them has no effect on CENP-A (Blower and Karpen, 2001; Collins et al., 2005; Liu et al., 2006; Regnier et al., 2005). Finally, overexpression of CENP-A results in its mislocalization to noncentromeric regions and the formation of ectopic kinetochores at these sites (Heun et al., 2006). Collectively, CENP-A is the epigenetic mark of centromeres that establishes and propagates centromeric identity.

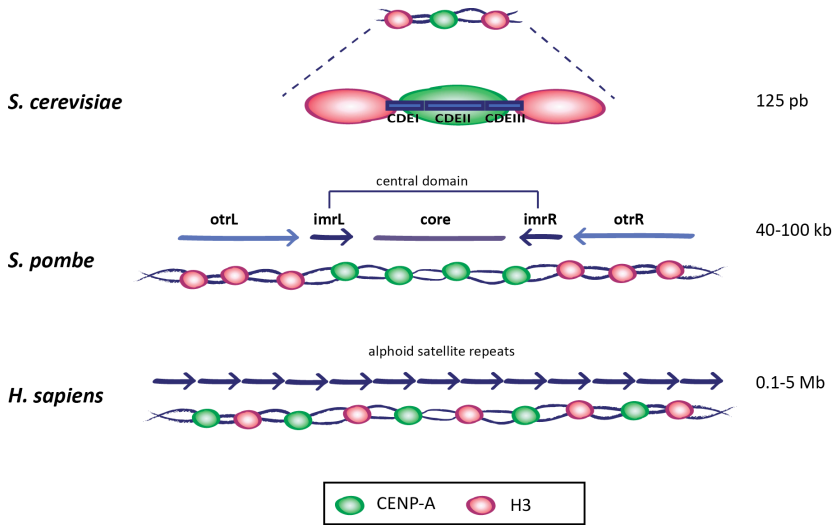


Figure I2. Centromere organization in different organisms.
Organization of centromeric DNA in budding yeast, fission yeast and humans.

Evidence supporting the epigenetic nature of centromere propagation comes from the discovery of two unusual centromere formations, pseudodicentric chromosomes and neocentromeres. Pseudodicentric chromosomes contain two discrete regions of a-satellite DNA but only one of them will form an active centromere, showing that the presence of a-satellite sequence is not enough to form a functional centromere (Schueler and Sullivan, 2006). Consequently, CENP-A is only recruited to the active centromere, while CENP-B recognizes and localizes to both centromeric regions, due to the presence of CENP-B box in both of them (Earnshaw et al., 1989, Sullivan and Schwartz, 1995; Warburton et al., 1997). Neocentromeres are functional centromeres formed *de novo* ectopically on noncentromeric chromatin sites. Although these regions lack centromeric a-satellite sequences, they are completely capable to recruit all centromere proteins, starting by CENP-A, and assemble a functional kinetochore (Marshall et al., 2008; Basset et al., 2010; Cleveland et al., 2003). Therefore, stable neocentromeres and pseudodicentric chromosomes demonstrate that DNA sequence is not the main determinant of centromere identity.

2.1.3 CENP-A is a variant of histone H3

CENP-A and H3 share a 60% sequence homology exclusively in carboxy-teminal part where they both have a histone fold domain (HFD) while their N-terminal tails show high divergence. Furthermore, N-terminal part of CENP-A possesses the flexibility typical for histones, although its sequence differs from all other histones. Also, the size and sequence of CENP-A highly differs among diverse organisms (Henikoff et al., 2005; Malik and Henikoff, 2003, Talbert et al., 2002, 2004; Hirsch et al., 2009; Quenet and Dalal, 2012). Nonetheless, *in vitro* experiments showed that CENP-A could replace H3 in reconstituted octameric nucleosomes, suggesting that overall structure of centromeric and canonical nucleosome was likely to be similar (Yoda et al, 2000). When of histone H3 HFD was replaced with that of CENP-A, H3 was recruited at centromeres, suggesting it is the structural element necessary for centromeric localization (Sullivan et al., 1994). CENP-A nucleosomes have a more rigid conformation than H3 nucleosomes. The domain responsible for such difference are in the first loop (L1) and second alpha helix (a2) of HFD, where CENP-A interacts with histone H4 (Figure I3A). If this region is exchanged with the corresponding one in H3 nucleosomes, H3 nucleosomes will be exclusively deposited at centromeres (Black et al., 2004; 2007). Hence, this region and is named the centromeric targeting domain or CATD. The CATD not only directs the association of CENP-A to centromeres but also makes a structure that enables its recognition by specific factors and in this way propagates epigenetic mark from one generation to another (Zhou et al., 2011, Sekulic et al., 2010).

2.1.4 Composition of CENP-A nucleosomes

The composition of CENP-A nucleosomes has been controversial (Padaganeh et al. 2013). The question under debate is whether the CENP-A particle comprises one molecule each of CENP-A and histones H4, H2A and H2B (forming a tetramer or hemisome) or two molecules of all four histones (forming an octamer) (Figure I3B). Nucleosome reconstitution experiments showed that octameric CENP-A nucleosomes can be efficiently assembled *in vitro* (Camahort et al, 2009; Furuyama et al, 2013). The crystal structure of CENP-A nucleosomes reconstituted *in vitro* from purified core histones revealed octamers containing two copies of each histone (Tachiwana et al, 2011). However, budding yeast Cse4 *in vitro* reconstitutes in the form of hemisomes (Furuyama et al, 2013). Moreover, CENP-A nucleosomes immunoprecipitated from *Drosophila* and human cells display half the height of bulk histone H3 nucleosomes by atomic force microscopy (AFM) (Dalai et al, 2007). Miell et al (2013) have now used AFM to show that octamers assembled with H3 containing CENP-A CATD also have reduced size, which might explain why CENP-A nucleosomes show the same feature. CENP-

A nucleosomes seem to wrap 110-150 bp of a satellite DNA in vivo, which would be too long for tetramer nucleosome (Hasson et al, 2013). Using a new fluorescent imaging approach to count the number of CENP-A molecules per mononucleosome at endogenous human centromeres also demonstrates that they contain two CENP-A molecules confirming the octameric model (Padaganeh et al., 2013).

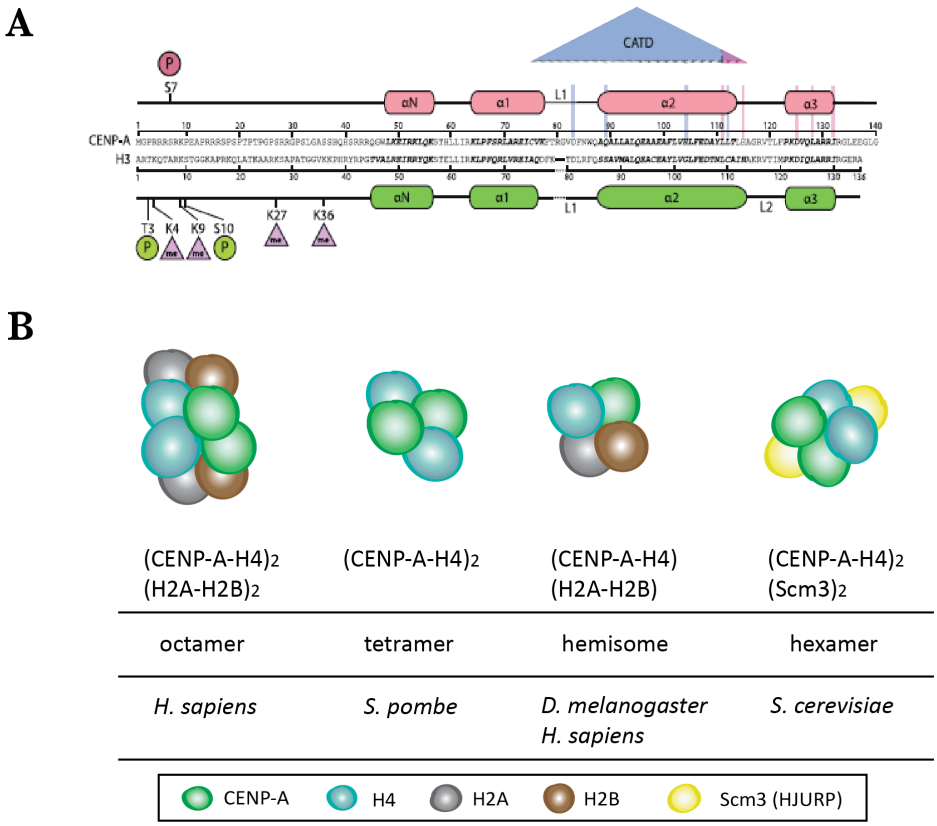


Figure I3. Centromere organization in different organisms.

A) Secondary structure elements of histones CENP-A and H3. CATD is the CENP-A targeting domain. Image taken from Stellfox et al. (2012).

B) Models of different types of CENP-A nucleosomes. CENP-A nucleosomes can form octamers in which CENP-A replaces H3, as it was recently show for humans. In *S. pombe* tetramers are formed containing only CENP-A and H4 histones. In *Drosophila* and humans, the existence of hemisomes was detected, which are formed from one CENP-A/H4 dimer and one H2A/H2B. Ultimately, in *S. cerevisiae*, Scm3 replaces H2A/H2B and forms a hexamer.

2.1.5 Features of centromeric chromatin

Although specified by the presence of CENP-A nucleosomes, centromeric chromatin also contains H3 nucleosomes. Chromatin stretching experiments revealed that centromeric chromatin is organized in blocks of CENP-A nucleosomes interspersed with blocks of H3 nucleosomes (Blower et al, 2002; Figure I4). Two different models have been proposed to explain how centromeric chromatin is organized. First one suggests that, despite their linear proximity, CENP-A and H3 occupy spatially distinct regions within mitotic chromosomes. CENP-A nucleosomes are located on the outward side of the mitotic chromosome while H3 nucleosomes reside inwards, within the inner chromatid region. This organization ensures that CENP-A is exposed on opposite sides of sister centromeres to promote formation of kinetochores that then interact with microtubules from opposite poles (Allshire and Karpen, 2008; Blower et al., 2002; Marshall et al., 2008). A newer model proposes that centromeric chromatin folds to make planar sinusoidal layers which then results in interspersed CENP-A and H3 nucleosomes both exposed towards the outer kinetochore (Ribeiro et al., 2010). Nonetheless, higher-order structure of centromeres, as well as the mechanisms that drive the organization of centromeric chromatin, remain largely unknown.

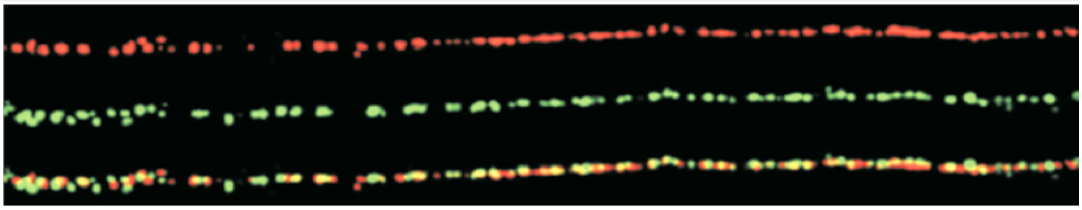


Figure I4. Interspersed H3 and CENP-A nuclosomes in human centromeres.
Extended chromatin fibers feom human cells stained with CENP-A (red) and H3 (green) antibodies. Images taken from Blower et al. (2002).

Centromeres are normally embedded in large domains of closed, inactive chromatin, called pericentric heterochromatin. In *Drosophila* and human cells, however, centromeric H3 contains dimethylation of lysine 4 (H3K4me2), a modification associated with open but not actively transcribed chromatin. Additionally, marks of silent (acetylation of histones H3 and H4) as well as actively transcribed chromatin (H3K9me3) were not present in centromeric chromatin (Sullivan and Karpen, 2004). This specific combination of posttranslational modifications on centromeric chromatin might be important for the maintenance of centromeric region and assist the CENP-A deposition or prevent its incorporation in noncentromeric sites. Interestingly, if CENP-A is overexpressed in *Drosophila* S2 cells it will

preferably incorporate in regions between euchromatin and heterochromatin, and form an ectopic kinetochore (Olszak et al., 2011; Heun et al., 2007; Mendiburo et al., 2012).

2.1.6 Cell cycle regulation of CENP-A assembly

In order to maintain the centromeric identity CENP-A nucleosomes have to be deposited onto centromeres in each cell cycle. Conventional histones are synthesized during S phase (Heintz et al., 1983) and assembled into chromatin in a replication-coupled manner (Worcel et al, 1983). On the other hand, histone variants are assembled in a replication-independent manner (Ahmad and Henikoff, 2001; 2002, Groth et al., 2007; Shelby et al., 2000; Tagami et al., 2004) and aided by specific histone chaperons and assembly factors that regulate their deposition at specific chromosomal loci (Groth et al., 2007; Polo and Almouzni, 2006).

CENP-A is synthesized in G2 phase and it is assembled into chromatin independently of DNA replication (Shelby et al., 2000). During S phase, parental CENP-A nucleosomes are equally distributed to daughter strands of DNA (Hemmerich et al., 2008; Jansen et al., 2007; Mellone et al., 2011; Schuh et al., 2007; Figure I5). The nucleosome-free gaps, formed after CENP-A nucleosome distribution, are filled with H3 histone variants, H3.1 and H3.3. However, it seems that only histone H3.3 acts as a placeholder for the CENP-A nucleosomes that will be incorporated in the subsequent assembly process (Dunleavy et al., 2011).

In *S. cerevisiae* the assembly of CENP-A nucleosomes occurs during the S phase (Pearson et al., 2004). In *S. pombe*, CENP-A levels reach a maximum before the canonical histones in early S phase and CENP-A is loaded both during S and G2 phase (Takayama et al., 2008). In *Drosophila* early embryos, CENP-A is incorporated in anaphase (Schuh et al., 2007), while in *Drosophila* cells incorporation occurs during metaphase (Ahmad et al., 2001; Mellone et al., 2011). In human cells, CENP-A incorporation occurs in early G1 phase although it requires passage through mitosis (Jansen et al., 2007). The reason for this requirement is unclear.

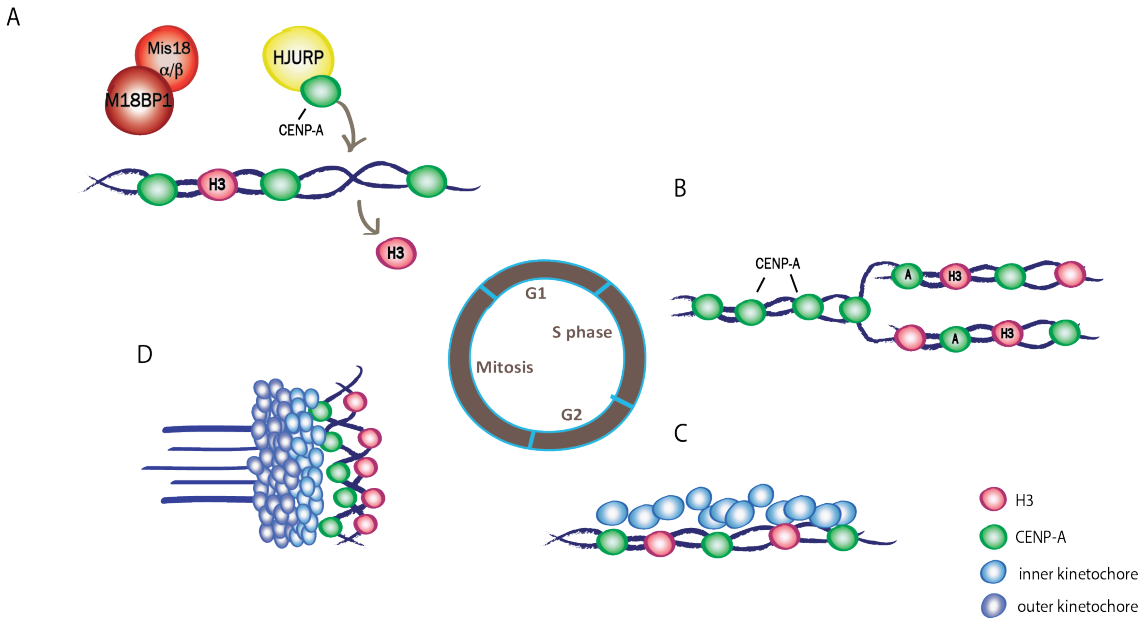


Figure I5. CENP-A deposition occurs upon exit from mitosis and entry into G1.

CENP-A nucleosomes are incorporated onto centromeric chromatin in M to G1 transition by the act of HJURP and Mis18 complex (A). In interphase during DNA replication CENP-A nucleosomes get diluted and interspersed with H3 nucleosomes (B). Some inner kinetochore proteins start to localize at centromeres in late interphase (C), while in mitosis the whole kinetochore is assembled upon the centromere.

2.1.7 Factors involved in CENP-A assembly

HJURP is a CENP-A specific chaperone

The Holliday junction recognition protein, HJURP, was first identified in the affinity purifications of soluble CENP-A complexes in human cells as a component of pre-nucleosomal CENP-A-H4 complex and is required for incorporation of new CENP-A (Dunleavy et al., 2009; Foltz et al., 2009). *Xenopus* HJURP performs the same function and is stored in the egg cytoplasm associated with CENP-A (Bernad et al., 2011). In both systems HJURP targeting depends on the members of the Mis18 complex (Barnhart et al., 2011; Moree et al 2011). Functional homologues of HJURP (named Smc3) have been characterized in budding and fission yeast (Camahort et al, 2007; Mizuguchi et al, 2007; Stoler et al., 2007; Williams et al., 2009; Pidoux et al., 2009; Sánchez-Pulido et al., 2009). In Drosophila a protein not related to HJURP named CAL1 is required for CENP-A deposition (Erhardt et al., 2008; Mellone et al., 2011)

HJURP functions as a specific CENP-A chaperone. It binds directly and specifically to the CATD domain of CENP-A through its CENP-A binding domain (CBD) (Foltz et al, 2009; Shuaib et al, 2010). HJURP CBD facilitates the deposition of CENP-A-H4 tetramers onto plasmid DNA *in vitro* (Shuaib et al., 2010). A slightly longer N-terminal fragment of HJURP ($HJURP^{Scm3}$) efficiently assembles octameric CENP-A nucleosome *in vitro,* but not H3 nucleosomes (Barnhart et al., 2011). HJURP binding also blocked CENP-A-H4 association with DNA, consistent with its function as a chaperone and suggesting that HJURP association may prevent unspecific binding to DNA (Hu et al., 2011). Artificially tethering HJURP to a non-centromeric site led to CENP-A incorporation and formation of a functional ectopic kinetochore, and bypassed the requirement for M18BP1 (Barnhart et al., 2011).

The Mis18 complex primes chromatin for CENP-A loading

The earliest recognized step in the recruitment of CENP-A nucleosomes is the association of Mis18 complex, which localizes to centromeres in late anaphase (Fujita et al., 2007). Mis18 was first identified in a screen for chromosome segregation mutants (Hayashi et al; 2004). Sequence homology searches led to the identification of Mis18α and Mis18β in human cells, and a third interactor named Mis18-binding protein (M18BP1; Fujita et al., 2007). Depletion of any of the three proteins by RNA interference (RNAi) showed that they depend on each other for proper localization to the centromeres and all are needed for deposition of newly synthesized CENP-A at centromeres (Fujita et al, 2007; Hayashi et al; 2004). Knocking down M18BP1 in *C. elegans* or in *X. laevis* egg extracts also prevents the assembly of CENP-A at centromeres (Maddox et al., 2007; Moree et al., 2011). However, a direct interaction between Mis18 with CENP-A has not been observed so far in any of the studied organisms. Evidence form yeast originally suggested the Mis18 complex could modify chromatin to make it accessible for CENP-A nucleosomes (Fujita et al., 2007). More recently, Mis18α has been shown to participate in maintaining DNA methylation at centromeric chromatin though recruitment of DNA methyltransefrases DNMT3A and DNMT3B. In the absence of Mis18α, the histone modification pattern of centromeric heterochromatin is altered in the centromeric region, which correlates with a decrease in non-coding transcripts from the satellite repeats and CENP-A deposition is abolished (Kim et al., 2012). In addition, the Mis18 complex seems to be important for the proper localization of CENP-A chaperone HJURP (Foltz et al., 2009).

Chromatin remodelers and other factors involved in CENP-A assembly and maintenance

Chromatin remodelers are large protein complexes that organize, mobilize and remodel nucleosomes in the wide range of chromosomal processes including transcriptional regulation and chromatin assembly. Recently, new functions for remodelers were revealed, such as deposition of histone variants, participation in cohesion function and RNA transcript elongation and termination. The chromatin remodeler FACT was first identified as a factor that facilitates chromatin transcription *in vitro* independently of ATP (Orphanides et al, 1998). FACT complex consists of two subunits, SPT16 and SSRP1, and it directly binds nucleosomes and H2A-H2B dimers. Interestingly, both subunits of FACT complex were found in CENP-A purifications from HeLa cells (Foltz et al, 2006; Obuse et al, 2006). Moreover, FACT was implicated in CENP-A nucleosome assembly in chicken DT40 cells (Okada et al, 2009). In this study, FACT was recruited to centromeres by the CENP-H/I/K/M complex and, in turn, it recruited ATP-dependent chromatin remodeling protein CHD1 to centromeres. CHD1 is required for the formation of centromeric chromatin in *S. pombe* (Walfridson et al, 2005) and DT40 cells (Okada et al., 2009) but not in Drosophila S2 cells (Podhraski et al, 2010).

Stabilization of CENP-A nucleosomes after their deposition involves the remodeling and spacing factor complex (RSF) which consists of two subunits, Rsf-1 and Snf2h (Perpelescu et al, 2009). Both RSF subunits were found in CENP-A immunoprecipitations from interphase cells (Izuta et al, 2006). Maintenance of newly assembled CENP-A also requires MgcRacGAP, a Rho family GTPase activating protein, the Rho family guanine nucleotide exchange factor Ect2 and the small GTPases Cdc42 and Rac (Lagana et al, 2010). MgcRacGAP downregulation caused a complete loss of newly assembled CENP-A from centromeres while parental CENP-A remained unaffected. In *Xenopus*, condensin II promotes deposition CENP-A nucleosomes and also prevents their eviction (Bernad et al, 2011).

A role for transcription in CENP-A assembly?

Tethering H3K4me2-specific demethylase LSD1 to the centromeric region of a human artificial chromosome (HAC) caused a decrease in centromeric transcription from the alphoid satellite DNA, failure to recruit HJURP and thereby a defect in the incorporation of newly synthesized CENP-A (Bergmann et al., 2011). Later, using the same HAC system, Bergmann et al., (2012) observed that tethering a potent transcriptional activator to centromeres to induce high levels of alphoid transcription resulted in rapid inactivation of the HAC while also impairing the CENP-A loading. Collectively, these results imply that centromeric transcription could have a role in CENP-A propagation and maintenance, but also suggest that in order to allow centromere function, this transcription has to be tightly

regulated. Recent experiments in HeLa cells have reported active transcription by RNA polymerase II at centromeres during mitosis (Chan et al., 2012). Transient inhibition of RNA pol II activity caused a decrease in centromeric transcripts, reduced CENP-C at centromeres and an increase in lagging chromosomes.

2.2 Constitutive centromere associated network (CCAN)

Since CENP-A nucleosomes are not sufficient for full kinetochore assembly (Van Hoser et al., 2001; Gascoigne et al., 2011), it was proposed that other factors might contribute in this process. Biochemical purification studies in human and chicken cells have identified a group of about 20 proteins that are localized at centromeres throughout the cell cycle and therefore termed constitutive centromere associated network (CCAN) (Foltz et al., 2006; Okada et al., 2006; see Figure I6). They are divided in two complexes. One is called the nucleosome associated complex (NAC) and consists of proteins CENP-B, CENP-C, CENP-T, CENP-W, CENP-H, CENP-M, CENP-N, CENP-I and CENP-U and associates with CENP-A nucleosomes. The other protein complex was isolated based on its association with the NAC proteins, was termed CENP-A nucleosome distal complex (CAD), and contains CENP-K, CENP-L, CENP-O, CENP-P, CENP-Q, CENP-R and CENP-S. CAD complex needs NAC proteins for proper localization to centromeres, while all of CCAN members seem to be necessary for the assembly of outer kinetochore proteins during mitosis. However, all of them are dependent on CENP-A for its assembly, except CENP-B, which recognizes centromeric B-box sequence (Foltz et al., 2006; Okada et al., 2006). Although the exact function of many of CCAN proteins remains to be elucidated, most of them seem to be important for proper kinetochore formation. Some have also been implicated in the propagation and maintenance of CENP-A nucleosomes. One of such is CCAN protein CENP-N that recognizes and interacts exclusively with CENP-A nucleosomes, but in a DNA sequence-independent manner (Carroll et al., 2009). It is stably associated with centromeres only in S and G2 phases, probably to re-establish the kinetochore (Hellwig et al., 2011). CENP-N depletion affects the recruitment of CENP-H, CENP-I and CENP-K, and partially CENP-C and CENP-A (Carroll et al., 2009). Downregulation of CENP-H/CENP-I complex that interacts with CENP-N also causes problems in the loading of new CENP-A (Okada et al., 2009).

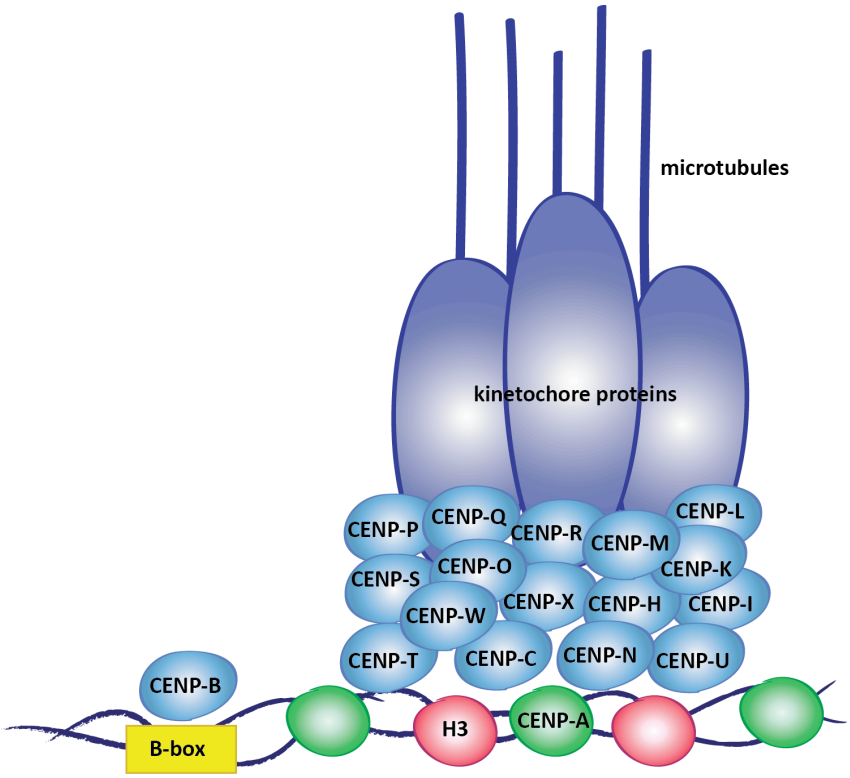


Figure I6. A model of CCAN proteins.

CCAN proteins localize directly upon centromeric chromatin interacting with either CENP-A nucleosomes or centromeric H3 nucleosomes. They serve as a platform for the assembly of outer kinetochore proteins such as the members of KMN network (Knl1 complex, Ndc80 complex and Mis12 complex) that bind to microtubules in mitosis.

2.2.1 Centromeric protein C (CENP-C)

CENP-C was one of the first identified centromeric proteins (Saitoh 1992). CENP-C is located closest to the CENP-A nucleosomes (Wan et al., 2009). It is also the last one to be lost before CENP-A nucleosomes are disrupted, when kinetochore inactivation is induced by tethering the KAP1 co-repressor to a HAC centromere (Cardinale et al, 2009). According to the experiments from HeLa and chicken DT40 cells, CENP-C directly interacts with centromeric H3 nucleosomes (Obuse et al, 2004; Hori et al, 2008). However, Carroll et al reported that CENP-C directly binds to the CENP-A nucleosome *in vitro* through its carboxyl terminal region (Carroll et al, 2010; Guse et al, 2011). CENP-A is required for proper recruitment of CENP-C to centromeres, while other CCAN components are differently needed depending on the system. In human cells the depletion of CENP-K leads to 50% depletion of CENP-C at centromeres (Hori et al, 2008) but CENP-C is able to localize to centromeres in the absence of CENP-I and CENP-H proteins. In chicken cells, CENP-C is

dependent on CENP-K/I/L/M for targeting to centromeres in interphase, but not in mitosis (Fukagawa et al, 2001; Nishihashi et al, 2002; Kwon et al, 2007).

CENP-C and particularly its amino terminal region are important for the proper recruitment of other CCAN members and kinetochore proteins (Figure I7). Depletion of CENP-C from *Xenopus* egg extracts affects the localization of other CCAN and kinetochore proteins CENP-K, CENP-E, Mad2, Rod and Mis12 (Milks et al, 2009). CENP-C downregulation by RNAi in HeLa cells caused a reduction in centromeric levels of CENP-T, CENP-I, CENP-K and CENP-H (Carroll et al, 2010), which is required for proper CENP-N localization (Foltz et al, 2006). Ectopic localization of CENP-C together with CENP-T proved to be sufficient for the recruitment of major kinetochore proteins and the assembly a functional kinetochore (Gascoigne et al, 2011; Hori et al, 2013; Kwon et al, 2007). Moreover, CENP-C seems to directly recruit Mis12 complex, a member of the kinetochore KMN network, through its N terminal region (Screpantti et al, 2011). The same is observed in *Drosophila* cells, where CENP-C was shown to interact with Nnf1, a subunit of the Mis12 complex, and the ectopic localization of CENP-C amino terminus induced an accumulation of KMN proteins (Przewloka et al, 2011). Therefore, CENP-C seems to be an essential protein for kinetochore recruitment and it provides a connection between centromeric chromatin and the microtubule binding components of the outer kinetochore. Consistent with this, depletion of CENP-C causes dramatic effects on chromosome alignment (Fukagawa and Brown, 1997; Fukagawa et al, 1999; Kwon et al, 2007).

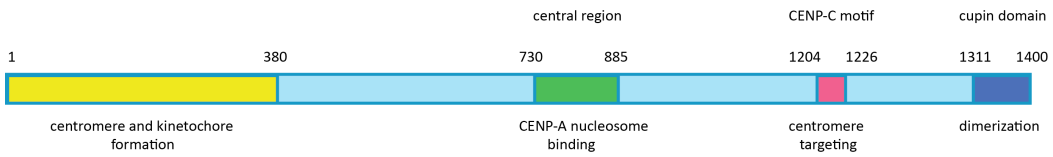


Figure I7. The structure of CCAN protein CENP-C.

Xenopus CENP-C is a 1400 amino acids big protein that contains three distinct domains. Central region provides the binding to CENP-A nucleosome, CENP-C motif is responsible for it centromere targeting. Cupin domain provides dimerization of CENP-C. The N-terminal part of CENP-C provides the connection to kinetochores.

In human cells, RNAi mediated depletion of CENP-C led to a 25%reduction of total CENP-A levels, which was consistent with a modest CENP-A assembly defect (Carroll et al., 2010). On the other hand, CENP-C knockout in chicken DT40 cells, did not affect the levels of CENP-A or new CENP-A assembly (Okada et al., 2006). However, ectopic targeting of CENP-C protein in DT40 cells along with the most of the kinetochore components, also

recruited CENP-A (Hori et al., 2013). Whether CENP-C has a role in CENP-A nucleosome assembly still remains to be seen. In *Drosophila*, CENP-C is essential for CENP-A assembly. CENP-C and CAL1 are interdependent for their localization to centromeres and interact directly (Erhardt et al., 2008).

2.2.2 Centromeric proteins T and W (CENP-T /CENP-W)

CENP-T was first discovered in purifications from HeLa cells as a CENP-A nucleosome-associated member of CCAN (Foltz et al., 2006). It has a histone fold domain in its carboxyl terminal region and a long tail in N-terminus (Hori et al, 2008; Suzuki et al, 2011; see Figure I8). CENP-W is a small protein entirely composed of the histone fold domain (Hori et al, 2008). CENP-T and CENP-W form a complex through their histone fold domains (HFD), which also possess the DNA binding activity. As a complex, they bind histone H3, but not CENP-A nucleosomes, in centromeric regions (Hori et al., 2008). More recently it was proposed that CENP-T/CENP-W forms a nucleosome-like stable heterotetramer with CENP-S/CENP-X complex (Nishino et al., 2012). The CENP-T-W-S-X complex also binds and supercoils DNA.

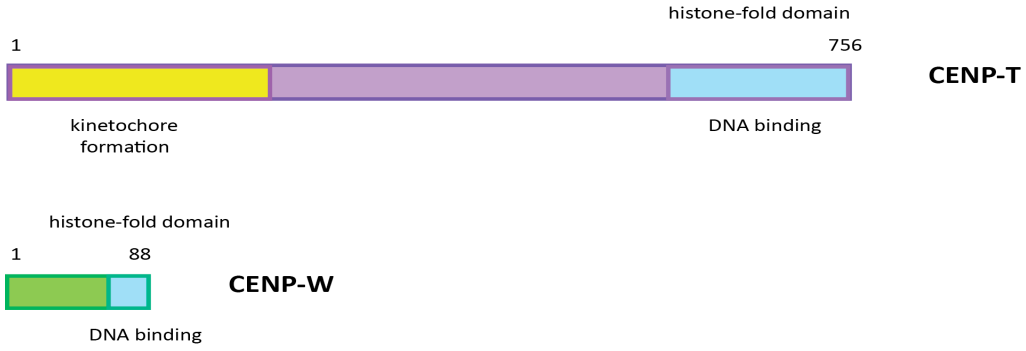


Figure I8. The structure of CCAN proteins CENP-T and CENP-W.
Schematic representation of the domains of CENP-T and CENP-W proteins from *Xenopus laevis*.

In HeLa cells, CENP-T/CENP-W complex assembles to centromeres through a dynamic exchange mechanism in late S phase and G2, and persists until chromosome segregation. It does not seem to be inherited by daughter cells like CENP-A, but it rather assembles onto centromeres again in each S phase (Prendergast et al, 2011). In chicken DT40 cells, CENP-T seems to be dependent for localization on CENP-H/I/K complex and CENP-

O complex and act in parallel with CENP-C (Hori et al., 2008; Okada et al., 2009). In human cells, CENP-C downregulation by RNAi differentially affects the localization of CENP-T (Carroll et al, 2010; Gascoigne et al, 2011). On the other hand, downregulation of CENP-T causes reduction or loss of most of the other CCAN components as well as increased number of cells arrested in mitosis and cell death (Hori et al., 2008). Ectopic targeting of CENP-T recruits most of the kinetochore proteins in HeLa cells (Gascoigne et al., 2011; Hori et al., 2013). This recruitment is mediated by the N-terminal tail of CENP-T that also specifically binds Ndc80 complex (Suzuki et al., 2011; Hori et al., 2013; Gascoigne et al., 2011). Phosphorylation of CENP-T by CDK is important for full kinetochore assembly in mitosis (Gascoigne et al., 2011; Gascoigne and Cheeseman, 2013). Whether the CENP-T and CENP-W have a role in CENP-A assembly remains to be tested.

2.3 *Xenopus laevis* egg extracts as a model system to study centromere assembly and function

Xenopus laevis egg extracts are a powerful model system as they can faithfully recapitulate many aspects of the cell cycle *in vitro*. For decades they have been used to study cell processes such as DNA replication, nuclear envelope breakdown and formation, spindle assembly, the regulation of cell cycle and specifically chromosome dynamics (Philpott and Yew, 2005; Desai et al., 1999; Murray, 1991). *Xenopus* laevis eggs are arrested in metaphase of meiosis II by the cytostatic factor (CSF) that inactivates the APC/C complex (Schmidt et al., 2006; Figure I9). Cytoplasmic extracts prepared by centrifugation from these *Xenopus* eggs maintain this arrest and are therefore called CSF extracts. Centrifugation separates egg yolk, pigments and lipids from the cytoplasm, which contains only proteins, and endogenous DNA is also lost in the process (Figure I10). However, it is possible to add exogenous DNA in the form of *Xenopus* sperm chromatin, to study processes occurring on chromatin (Desai et al., 1999). During spermatogenesis, most core histones are replaced with small, highly basic proteins called protamines, to facilitate chromatin compaction (reviewed in Grimes, 1986). However, in many animals, including *Xenopus*, CENP-A is quantitatively retained in sperm, appearing as discrete foci when observed by immunofluorescence (Haaf et al., 1990; Palmer et al., 1990; Zeitlin et al., 2005). This underlines the importance of CENP-A as a centromere marker and highlights the fact that the centromere is maintained at a specific locus through organismal generations.

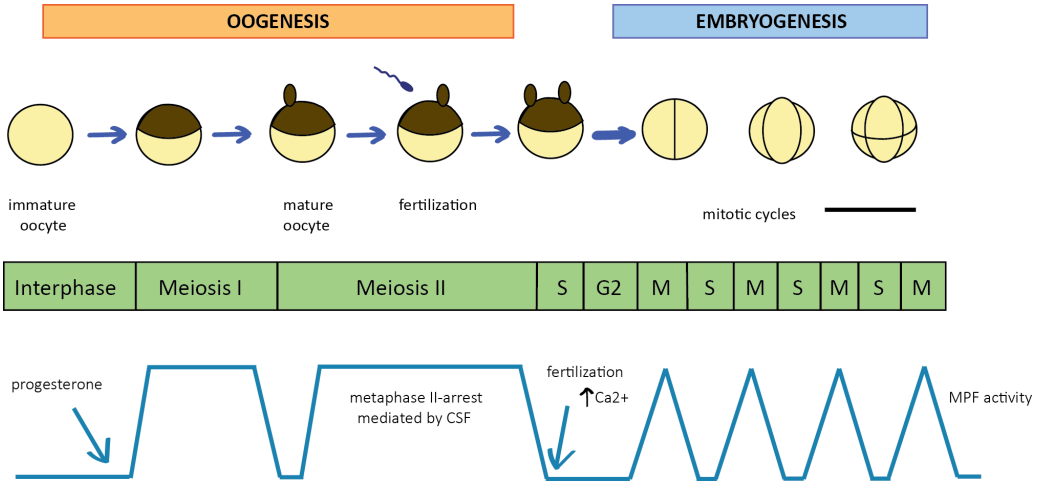


Figure I9. Oogenesis and early embryogenesis in *Xenopus* laevis.

Oocytes are born the same size as somatic cells and then arrested in G2 as they grow in diameter from 20 μm to 1 mm. Secretion of progesterone by the follicle cells surrounding the oocyte induces it to undergo meiosis I and enter meiosis II. The oocyte arrests in metaphase of meiosis II by the action of the CSF. Fertilization overcomes this arrest and initiates the early embryonic cells cycles that consist of rapid S phase followed by mitosis with no G phases, except in the first embryonic cell cycle.

When demembranated sperm is added to mitotic egg extracts, protamines are rapidly exchanged for core histones and chromosomes form condensed structures similar to condensed mitotic chromosomes observed within cells (Desai et al., 1997; Milks et al., 2009; Minshull et al., 1994; Philpott and Leno., 1992; Wood et al., 1997). When calcium is added to the extract to mimic fertilization, the cell cycle state of the extract transits from metaphase into interphase, chromosomes decondense and nuclear envelope forms around them (Emanuele and Stukenberg., 2009; Lohka and Maller., 1985; Murray, 1991). DNA replication begins shortly after release from metaphase arrest and it is completed at around 80 minutes following release (Desai et al., 1999; Sawin and Mitchison, 1991). Addition of more CSF extract or just cyclin B to the interphase extract, induces its cycling to the subsequent mitosis, which is followed by nuclear envelope breakdown, condensation of chromosomes and the assembly of functional kinetochore and spindle upon the centromere.

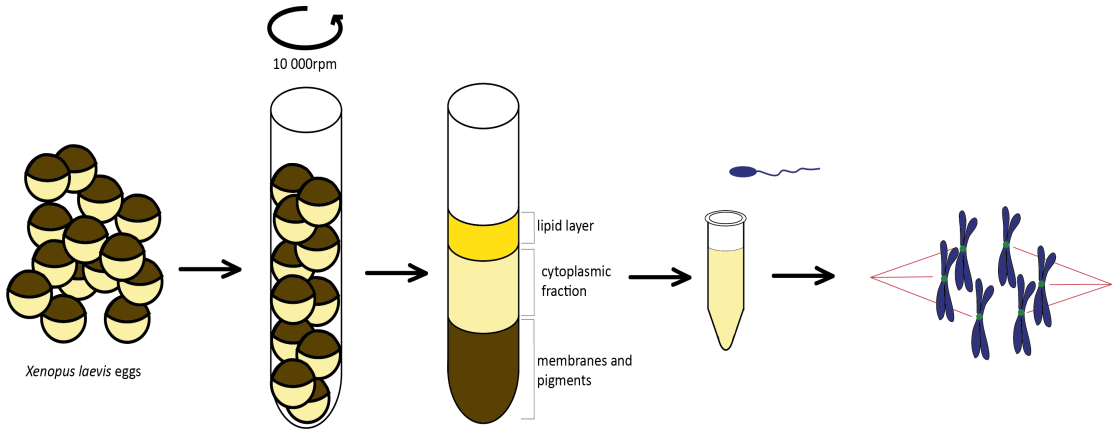


Figure I10. Preparation of *Xenopus laevis* egg extract.

The oocyte cytoplasm is isolated from arrested eggs by centrifugation. The yolk granules pellet and break open the cell. The cytoplasm is extracted from the middle layer using a syringe. Demembranated sperm chromatin is added as template. Addition of calcium overcomes CSF and the extracts cycle to interphase and subsequent mitosis. Many processes of chromosome and spindle dynamics can be recapitulated in the test tube.

There are many advantages in using *Xenopus* egg extracts as a model system. One is that the prepared extract is always completely synchronous, either mitotic or interphasic. Therefore, extracts can be used to reconstruct a defined cell cycle process *in vitro* and study it biochemically. Also, we study only one cell cycle without accumulating errors from previous cycles, which can arise in i.e. cells, and mask the roles of the studied proteins. Functional assays can be performed after elimination of a protein from an extract by depletion with specific antibodies. Later, the eliminated protein can be added back to the extract, either as a recombinant protein produced in bacteria or translated in vitro in rabbit reticulocyte lysates, or as mRNA also produced in vitro that can be translated by the extract. In this way, the specificity of the depletion can be tested. Moreover, addition of truncated or mutant version of the factor under study will allow dissecting its functional domains. One important feature of the extracts and that many proteins are stored in the egg associated with other factors forming complexes. In practical terms, this means that when we deplete one protein with specific antibodies we may be depleting all its associated factors as well. If these factors are important for the particular function we are addressing, they will have to be also added back in order to rescue the observed defect.

2.3.1 *In vitro* assay to study the new CENP-A deposition

The ability to manipulate the cell cycle state of extracts and to recapitulate centromere formation on sperm chromatin makes *Xenopus* egg extracts model system suitable for studying CENP-A assembly. Importantly, most centromere proteins and 54 known CENP-A assembly factors are conserved between humans and *Xenopus* (Bernad et al., 2011; Guse et al., 2011; Milks et al., 2009; Schmidt-Zachmann et al., 1987; Wade et al., 1998). To study the role of different proteins in CENP-A deposition we have developed a quantitative immunofluorescence-based assay to measure CENP-A incorporation in chromosomes assembled in *Xenopus* egg cell-free extracts. We quantify the change in the fluorescent signal of CENP-A at individual centromeres. Nuclei in two different time points of an assembly reaction, or under two different conditions, are spun over the same coverslip, and then processed for immunofluorescence and imaged together. On these images, we quantify the intensity of CENP-A signals and calculate the average increase in CENP-A staining for each pair of nuclei (see Materials and methods for details). Since sperm chromatin already contains CENP-A and new CENP-A is deposited upon exit from mitosis, the amount of CENP-A at centromeres in interphase nuclei in the control condition should be double than in CSF (mitotic) extract.

Using this assay we have previously shown that CENP-A incorporation occurs upon exit from mitosis but independently of DNA replication, same as in Drosophila embryos and human cells (Schuh et al., 2007; Jansen et al., 2007). Immunodepletion of proteins involved in the deposition of other histone H3 variants (CAF-1 and HIRA did not affect significantly the incorporation of CENP-A. As mentioned before, CENP-A deposition depends instead on *Xenopus* HJURP. The chaperone is stored in the oocyte cytoplasm forming pre-assembly complex with CENP-A. We also found that condensin II is also required for efficient CENP-A incorporation although the mechanism for this requirement is unclear (Bernard et al., 2011).

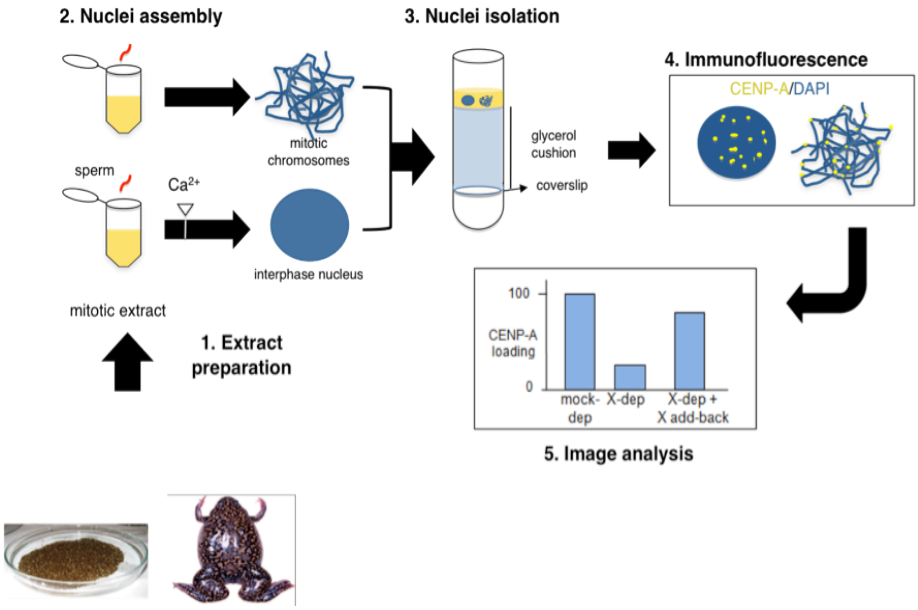


Figure I11. An assay to measure CENP-A incorporation in *Xenopus* egg extracts.

Extracts prepared from laid eggs are subjected to immunodepletion with specific antibodies against factor X (step 1). Sperm chromatin is added to mitotic extracts. In one tube (top) incubation proceeds for 80-120 min to get mitotic chromosomes. In another tube (bottom), calcium is added to drive entry into interphase 40 min after sperm addition. Incubation proceeds for 80 min to get interphase nuclei that had undergone replication (step 2). Equal volumes of the reaction mixtures in both tubes are combined, fixed and centrifuged over a coverslip placed at the bottom of a glycerol cushion. Coverslips are processed for immunofluorescence with an antibody against CENP-A and DNA is stained with DAPI. Images of a mass of mitotic chromosomes next to an interphase nucleus are acquired and CENP-A signals are quantitated (see Material and Methods for details). The relative CENP-A loading efficiency of a depleted extract with respect to a mock depleted extract (considered 100%) is calculated and represented. If depletion of a factor X prevents loading of CENP-A and the defect is rescued by adding back the factor to the depleted extract, we conclude that factor X is involved in CENP-A incorporation.

Objectives

3. Objectives

Centromeres are epigenetically defined by the special nucleosomes containing histone H3 variant called CENP-A. In order to have a proper segregation of chromatin, this epigenetic mark has to be maintained by new incorporation of CENP-A nucleosomes in each cell cycle. Given the close localization of constitutive centromeric associated network (CCAN) proteins CENP-C, CENP-T and CENP-W to CENP-A nucleosomes, we wanted to study which roles these proteins play in the process of CENP-A assembly and which in stabilization of CCAN components and CENP-A maintenance.
Therefore, the main objectives of this thesis were:

1. Characterization of *Xenopus laevis* CCAN proteins CENP-C, CENP-T and CENP-W.

2. Study of the role of CCAN proteins CENP-C, CENP-T and CENP-W in kinetochore assembly in the *Xenopus* egg cell-free system.

3. Study of the role of CCAN proteins CENP-C, CENP-T and CENP-W in the process of loading of new CENP-A.

4. Study of the role of chromatin remodeler FACT in CENP-A incorporation.

Materials and methods

4. Materials and methods

4.1 Antibody generation

For this study we produced rabbit polyclonal antibodies for a number of proteins using as antigens either recombinant proteins purified from bacteria or synthetic peptides coupled to keyhole limpet hemocyanin (KLH). Antigen injection and sera production was performed by Innovagen (Lund, Sweden).

Recombinant proteins:

Full-length cDNA of xCENP-T from *Xenopus tropicalis* was transferred from IMAGE clone 7793391 to pENTR vector (Gateway). A fusion protein with a 6xHis tag in the N-terminus (vector pDEST17) was expressed in E. coli BLB21 cells and purified as an insoluble protein in 8M Urea. The protein was then refolded by dialysis in decreasing concentrations of Urea (to final 0.25M).

Full-length cDNA of xCENP-W from *Xenopus laevis* was transferred from IMAGE clone 6323080 to pENTR vector and then to pDEST17. The 6xtagged CENP-W fusion protein was expressed in E. coli BLB21 cells, purified in 8M Urea and dyalized as above until completely remove the Urea.

An N-terminal fragment of *Xenopus laevis* CENP-C (aminoacids 207-296) cloned as fusion with GST tag (Milks 2009) was expressed in bacteria and purified in glutathione agarose.

Full-length cDNA of xMis18α from *Xenopus tropicalis* was transferred from IMAGE clone 5335361 to pENTR vector and then to pDEST17. The His-tagged protein expressed in E. coli BLB21 cells was purified as an insoluble protein. The protein was then partially refolded by dialysis to a final concentration of 2M Guanidine Hydrochloride.

Peptides: xM18BP1, CKLFRTDEPTVSDDD; xFACTp140, CGHAPLPNPSKKRKK; xFACTp87, CSSDDSSSDDSAQKD.

The crude sera that recognize a protein of the appropriate size in immunoblot analyses of egg extracts fractionated by SDS-PAGE were affinity purified. For that, antigen columns were prepared by coupling the antigen to Affigel 10 or Affigel15 resin (depending on the pI) as described in Sawin et al, 1992. Purified antibodies were dialyzed overnight against TBS and stored in aliquots at 4ºC. They were next tested for immunoprecipitation and immunofluorescence.

Additionally, we have used previously made antibodies indicated in Table 1.

Antibody	Provider/Reference
myc	Cell Signaling Technology
H3	Abcam
xHJURP	Bernad et al, 2011
xCENP-A	Rivera and Losada, 2009
B4 (embryonic histone H1)	Bernad et al, 2011
xRbAp48	Bernad et al, 2011
xCAP- D3	Bernad et al, 2011
xRad21	Losada et al, 1998
Ndc80	Cleveland et al, 2003
xRbAp48	Bernad et al, 2011
xCENP-T	made for this study
xCENP-W	made for this study
xCENP-C	made for this study
xFACTp140	made for this study
xFACTp87	made for this study
xMis18α	made for this study
xM18BP1	made for this study

Table M1. Antibodies used in this study.

4.2 Preparation of *Xenopus leavis* egg extracts

The extracts are prepared from the eggs collected after a night of ovulation. Female frogs are prepared for ovulation after three successive hormone injections, two times with gonadotropin from pregnant mare serum (PMSG) and once with human chorionic gonadotropin (HCG). Once the eggs are collected, they have to be processed for extract preparation. First, eggs are washed several ime wit MMR buffer MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0,1 mM EDTA, 5mM HEPES, pH 7.8). Next, the gelatinous layer around the eggs is eliminated by washing with 2% cysteine solution in MMR. Afterwards, the eggs are washed in XBE2 buffer (100 mM NaCl, 0,1 mM $CaCl_2$, 1 mM MgCl2, 10 mM HEPES, pH 7.7, 5 mM EGTA, 50 mM sucrose) while also eliminating the activated eggs. The eggs are crushed by centrifugation at 1500rpm for 60s, the excess buffer is removed and the eggs are fractionated by centrifugation at 10000rpm for 15min at 16ºC. After centrifugation, the top layer of lipids and the bottom layer of membranes and pigments are discarded and only the middle cytoplasmic fraction is recovered as low speed supernatant (LSS) to be used in experiments (Figure M1). If this extract is centrifuged for another 2h at 50000 rpm that removes the rest of the lipids, membranes and ribosomes, and we obtain a high-speed supernatant (HSS) extract (Figure M1). Since *Xenopus* eggs are arrested in metaphase II of the meiotic division by a cytostatic factor, we call this extract CSF extract.

Addition of 100mg/ml cyclohexamide and 0.5mM $CaCl_2$ drives the extract into interphase. After 90 min, the extract can be driven back to mitosis by addition of 1 volume of CSF extract.

4.3 Immunodepletion and addback experiments

Proteins of interest were removed from the extract using immunodepletion, performed by using Affiprep Protein A support (Bio-Rad Laboratories) as described previously (Losada et al., 1998). In brief, depletion of 100 µl of extract required one (xCENP-C) or two (xCENP-T, xCENP-W, xFACTp140, xCAP-D3) rounds of incubation with 50 µl of beads bound to 25 µg of antibody. Agarose beads are incubated with the antibody during 1h rotating at room temperature, and then left during the night at 4º C rotating after which they are washed 3 times with XBE2 buffer. The extract is added to the beads and after one or two depletion rounds of 45 min the depleted extract is recovered by successive centrifugations at 4ºC for 10s. The efficiency of the depletion is estimated by SDS-PAGE and immunoblot.
For addback experiments, the full-length cDNAs of CENP-T and CENP-W were cloned into pCS2myc vector and the corresponding myc-tagged proteins were obtained using the TNT Quick Coupled Transcription/Translation system (Promega). The proteins were diluted in the extract up to 1:10 to replenish endogenous levels of the protein. Full length CENP-C cloned in pCS2myc was obtained from A. Straight (Moree et al, 2011). The vector was linearized with SalI and used for the production of mRNA using mMESSAGE mMACHINE SP6 transcription kit (Ambion). Normally, 0,5-1 mg/ml of final concentration of mRNA was used per experiment.

4.4 RNase treatment and RNA polymerase II inhibition

To destroy all RNA in the extract, RNase A (Roche) was used in two different final concentrations, 40ug/ml and 100ug/ml. The extract was treated for 30min at 22ºC before the sperm nuclei were added and the CENP-A loading assay was performed.
In the case for RNA polymerase II inhibition, extract was treated for 20min at 22ºC with two concentrations of a-amanitin (10ug/ml and 100ug/ml final concentration) to specifically inhibit RNA pol II. Also, actinomycin D was used in two concentrations (2ug/ml and 10ug/ml final concentration) as a more general inhibitor of transcription and replication.

4.5 Immunofluorescence

Chromosomes and nuclei assembled in *Xenopus* extract are processed for immunofluorescence in the following way. First, they are fixed by addition of 10 volumes of 2% paraformaldehyde in XBE2 with 0,25% of Triton X-100. After 10 min, the fixed assembly mixtures are spun down on coverslips through a 5 ml-cushion of 30% glycerol in XBE2 during 15 min at 6500xg at 4ºC. Coverslips are washed and then blocked for 1h in 3% BSA in TBST buffer (blocking solution) and then incubated for 2 hours with primary antibodies diluted in blocking solution at 2mg/ml in the case of xCENP-A, xCENP-T, xCENP-C, xNdc80; 5ug/ml for xFACTp140, xHJURP, xCENP-W. Secondary antibodies are diluted 1:100 in blocking solution and applied to the coverslips for 2 hours. In some experiments in which we need to use two rabbit antibodies, one of them is fluorescently labeled (Alexa 549-conjugated anti-xCENP-A). In this case, after incubation with the primary, unlabeled antibody followed by incubation with the secondary antibody, free rabbit IgG epitopes must be blocked with 1mg/ml non-immune rabbit IgG for 1 hour. Then, the labeled xCENP-A antibody is applied. In the end, DNA is stained with DAPI (1mg/ml), and the coverslips are mounted with Mowiol. Samples are analyzed with the Leica DM6000 microscope; black and white images are taken with a CCD camera and later processed with Photoshop.

4.6 CENP-A loading assay

The CENP-A loading assay is based on measuring the relative intensity of CENP-A between two samples of the same reaction of assembly taken at different times. In the majority of experiments the procedure is as follows: we assemble *Xenopus* mitotic chromosomes in CSF extract and incubate them for 90min at 22ºC, which will later serve as a control since sperm chromatin already contains CENP-A. Chromosomes are also assembled in CSF extracts that are mock immunodepleted or immunodepleted from a protein of interest by adding sperm nuclei (800-1000 nuclei/ml) to them and incubating it for 45min at 22ºC. Next, both samples are converted to interphase by addition of 100mg/ml cyclohexamide for 5 minutes at room temperature, that will that inhibits the production of cyclin B, and then 0.7 mM $CaCl_2$ to drive interphase. Also, biotin-16-dUTP (Roche) is added at 10 µM to mark the replicated nuclei and extract is incubated for 90-120min at 22ºC until obtaining interphase nuclei. Both samples, CSF control nuclei and interphase treated nuclei are mixed with 5 volumes of 0,25% Triton X-100 in XBE2 during 5 minutes and are fixed with two volumes of 0,4% paraformaldehyde in XBE2 with 0,1% of Triton X-100 during 15min. Next, they are spun down through glycerol cushion on the same coverslip. Immunofluorescence is done using a

specific xCENP-A antibody as primary antibody, donkey anti-rabbit FITC as secondary antibody and a Cy3-streptavidin to recognize the biotinylated nucleotides incorporated during replication. Coverslips are mounted using Mowiol. Samples are analyzed in a Leica DM6000 microscope. Photos of pairs of nuclei, one in mitosis and another in interphase are taken (Figure R). Quantification and processing were done with the ImageJ software (http://rsb.info.nih.gov/ij/). The intensity of CENP-A signals of interphase nuclei is measured compared to CSF nuclei and the obtained number is relativized to percentage of CENP-A loading. In the case of mock immunodepleted extract the obtained number would be 100% of CENP-A loading while for the other extracts that number could vary (Figure M2). The analysis is described below.

4.7 Image analysis for the CENP-A loading assay

For imaging, chromatin states were elucidated by morphology (condensed chromosomes vs. interphase nuclei) and/or biotin-dUTP incorporation (replicated vs. non-replicated nuclei). In most experiments, undepleted CSF-assembled chromosomes were used as standard reference. Images were taken using a microscope (model DM6000; Leica), with a HCX PlanApochromat 63×/1.4 oil immersion objective. Quantification and processing were done with ImageJ software (http://rsb.info.nih.gov/ij). Images of CENP-A staining were set to binary using the Threshold tool, and then individual centromeres were selected as regions of interest (ROIs) applying the Analyze Particles function. When required, the Watershed function was used to separate centromeres in close vicinity. Alternatively, centromeric regions were selected manually using drawing tools. Using ROI Manager, ROI lists were created for individual nuclei. The average Integrated Density (ID = average pixel intensity × area) was first calculated from the IDs of centromeres within each nucleus and then a ratio between the IDs of each imaged pair of nuclei was obtained. Finally, the average ID ratio (IDr) of at least 15 pairs of nuclei was calculated. The relative CENP-A loading efficiency of a depleted extract (D) with respect to a mock-depleted, control extract (C) is calculated as (IDrD-1)/(IDrC-1). In this case, we imaged together and quantified the CENP-A signals of nuclei assembled in CSF and late interphase. To measure CENP-A eviction, the average integrated density of CENP-A signals in chromosomes assembled in depleted CSF extracts, a reference sample was prepared in undepleted interphase. The resulting values are expressed as a ratio relative to the average value obtained for the mock-depleted extract.

4.8 Biochemical analysis of chromatin isolated from in vitro extract

Chromatin is assembled upon in vitro incubation of *Xenopus* sperm nuclei (1600-2000 nuclei/ml) in CSF LSS extracts depleted from proteins of interest or control extracts, which are supplemented with ATP energy mix (1mM MgATP, 10mM creatin phosphate, 50 µg/ml creatin kinase). After 40 minutes of incubation, part of the sample (about 80ul) is converted into interphase by adding 100ug/ul of cyclohexamide and 0,7 mM of CaCl2. After 90-120 minutes of incubation, part of the sample (about 40ul) is converted to mitosis by the addition of cyclin D90, non-degradable cyclin B, for another 90-120 minutes (Figure M1). Chromatin isolation is done by diluting the 40ul of samples with 10 volumes of XBE2 with 0,25% Triton X-100 for 10minutes on ice. Next, the diluted samples are centrifuged over cushion of 30% sucrose in XBE2 at 10000 x g for 15 minutes at 4°C. The samples are washed several times with XBE2 and prepared for analysis with SDS-PAGE and immunoblot.

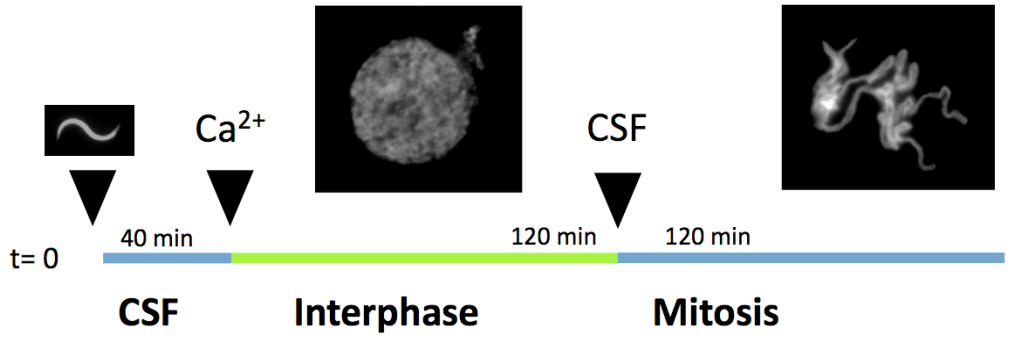


Figure M1. Chromatin assembly in *Xenopus* extract.
Sperm chromatin added to CSF extract condenses during the 40 minutes of incubation. When calcium is added to the extract to pass to interphase, chromatin decondenses which is visible under the microscope when DAPI is added to the aliquot of the reaction. After 120 minutes, extract is converted to mitosis and chromatin is again condensed.

4.9 Immunoprecipitation

For immunoprecipitation from *Xenopus* LSS or HSS extracts, 2-5 µg of antibody is added for 100 µl of the extract. The mixture is incubated during 2h on ice and then 10 µl of Protein Agarose A beads (Invitrogen) is added to it. The new mixture is incubated for 1h rotating at 4ºC. Afterwards, it is centrifuged at 5000-6000 rpm during 1 min, the supernatant that does not contain the protein of interest is recovered as a flow-through phase (FT), and the beads are washed several times with XBE2 buffer. Finally, the beads containing the immunoprecipitation fraction are resuspended in loading buffer to be later analyzed on immunoblot. As a control of immunoprecipitation, beads bound to rabbit IgG antibody are used.

4.10 Extract fractionation on a sucrose gradient

A high-speed supernatant (HSS) extract was overlaid on a 5 ml sucrose gradient (5%–20% made with XBE2), which was then spun at 36,000 rpm for 15 hr at 4°C in a SW50.1 rotor (Beckman). Eighteen fractions were taken manually, TCA-precipitated, and separated by SDS–PAGE and analyzed by immunobloting. A mixture of standard proteins in the same volume of XBE2 was centrifuged in parallel, eighteen fractions were taken manually, separated by SDS–PAGE and the gels were stained with Coomassie blue. The proteins used were thyroglobulin (8.5 nm, 19.4 S), catalase (5.2 nm, 11.3 S), ovalbumin (3.1 nm), and bovine serum albumin (BSA; 4.5S).

Results

5. Results

5.1 Role of CCAN proteins CENP-C, CENP-T and CENP-W in centromere propagation and function in *Xenopus* egg extracts

5.1.1 Biochemical characterization of CENP-C, CENP-T and CENP-W in the soluble egg extract

To characterize the CCAN proteins CENP-C, CENP-T and CENP-W in *Xenopus* egg extracts, we produced specific antibodies against each of them using as antigens synthetic peptides or protein fragments produced in bacteria (see Materials and Methods, section 7.1). By means of immunoprecipitation experiments, we found that CENP-T is present in the immunoprecipitates obtained with CENP-W antibodies, and viceversa (Figure R1A). This is consistent with previous reports indicating that CENP-T and CENP-W form a complex in chicken DT40 cells as well as in human cells (Hori et al, 2008). Unexpectedly, we also detected CENP-C in the CENP-W immunoprecipitates but not in the CENP-T immunoprecipitates. This suggests that the egg extract contains two distinct populations of CENP-W protein, one in complex with CENP-T and other with CENP-C. To have an idea of the relative abundance of these two populations, we depleted the extract from CENP-C, CENP-T and CENP-W proteins and asked how this depletion affected the other two proteins. When CENP-T-is depleted up to 90-95% from the extract, the levels of CENP-W are also reduced up to 80% whereas, as expected from the immunoprecipitation results, CENP-C is not affected (Figure R1B). CENP-T levels are still quite high in a CENP-W-depleted extract., but CENP-C levels decrease. The low signals obtained in the immunoblots of CENP-W makes it difficult to asseess the efficiency of the depletion in this case. Depletion of CENP-C only affects CENP-W levels mildly and does not reduce CENP-T (Figure R1B). Taken together, these results suggest that the majority of CENP-W protein is stored in the soluble egg extract in a complex with CENP-T, whereas a minor fraction (maybe around 20%) is associated with CENP-C. In addition, a fraction of CENP-T appears to be present in the extract either alone or associated to some other protein.

To further confirm these results, we performed a sucrose gradient fractionation of a total egg extract (Figure R1C). CENP-T indeed exists in two major forms with sedimentation coefficients around 5S and 9S that likely correspond to CENP-T alone and CENP-T in a complex with CENP-W, respectively. Most CENP-W is present in the extract associated with CENP-T (9S complex) and a very minor fraction associates with CENP-C in a complex with a sedimentation coefficient around 14-15S.

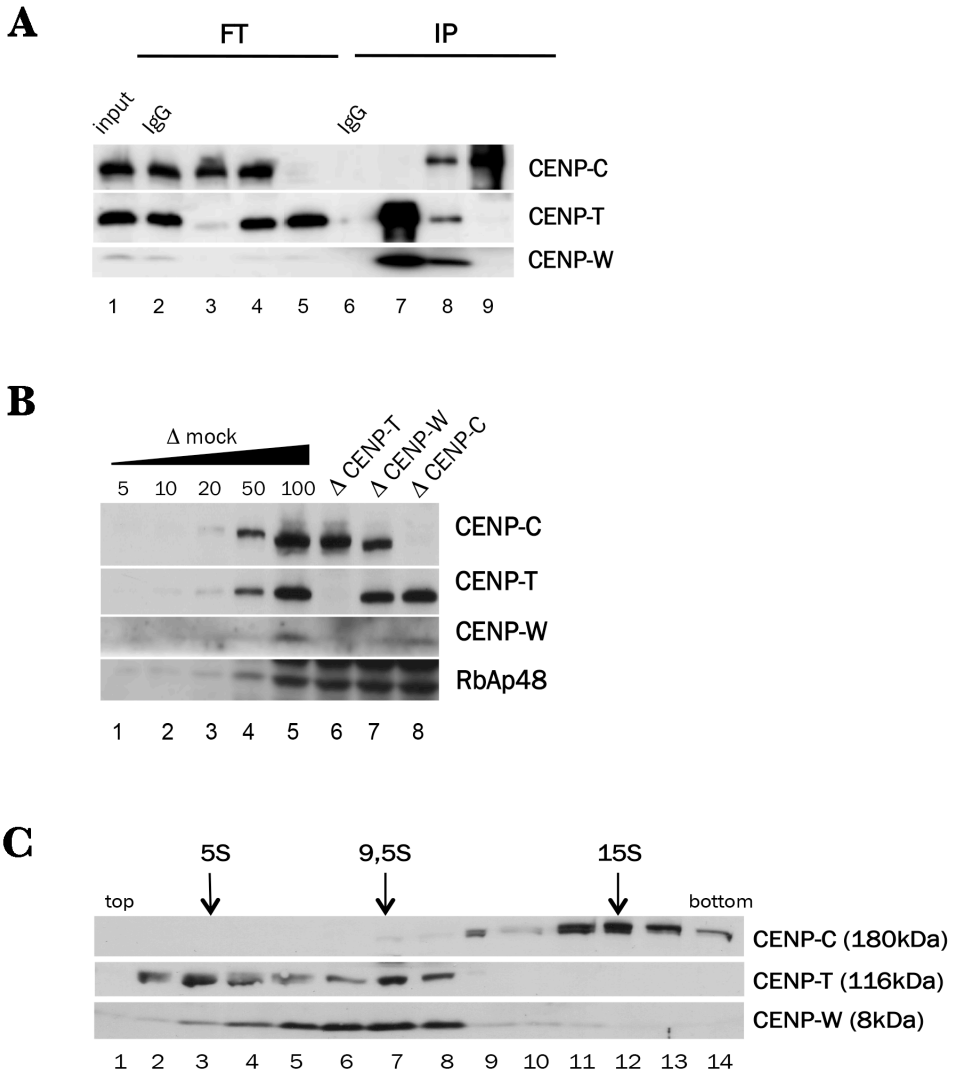


Figure R1. Distinct association of CENP-C, CENP-T and CENP-w in the soluble egg extract.

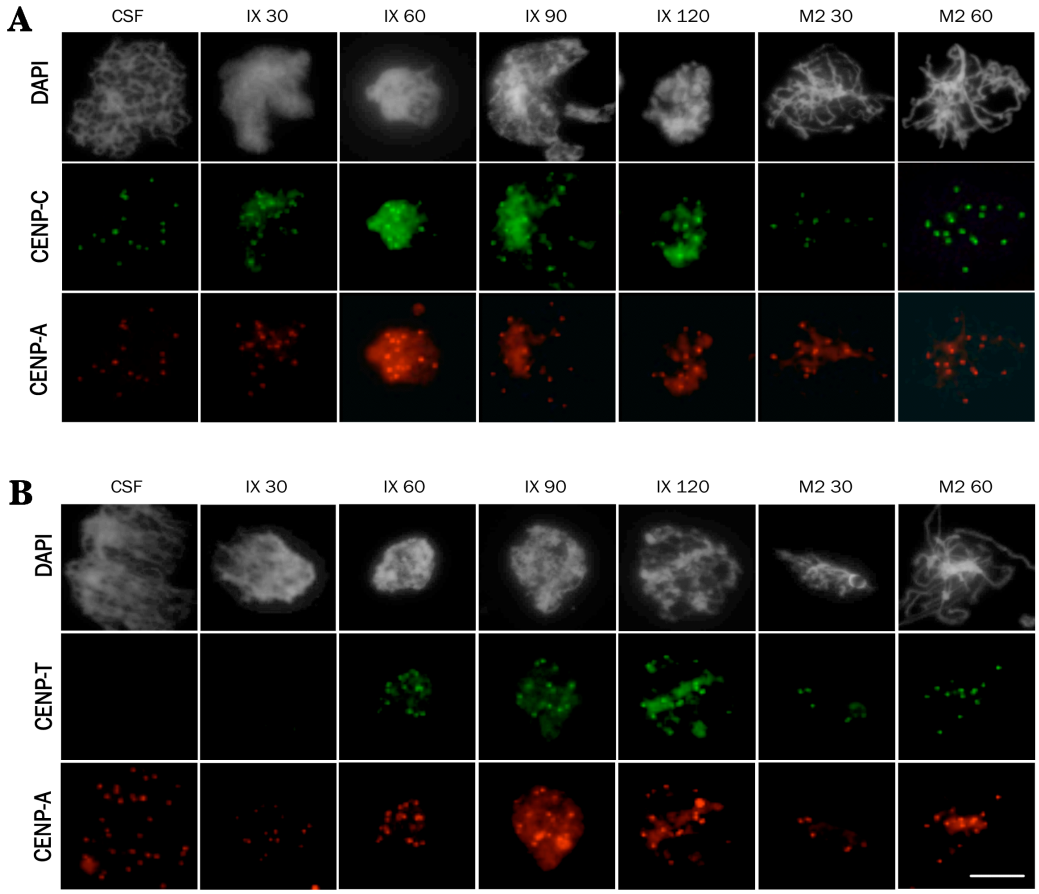
A) Immunoprecipitates (IP) obtained from *Xenopus* HSS extract with antibodies against CENP-C, CENP-T and CENP-W were analyzed by immunoblot. Immunoprecipitation with non-immune rabbit IgG was used as control. Aliquots of the flow through (FT) from each reaction and of the input extract were also analyzed.

B) Immunoblot analysis of 1.5-µl aliquots of extracts depleted with specific antibodies (Δ) alongside increasing amounts of a mock-depleted extract (expressed as percentage of a 1.5-µl aliquot). RbAp48 is used as a loading control.

C) A soluble egg extract was fractionated on a sucrose gradient (5-20% sucrose) and analyzed by immunoblot. The sedimentation coefficients of the major peaks of CENP-T, CENP-W and CENP-C are indicated.

5.1.2 Centromeric targeting of CENP-C, CENP-T and CENP-W throughout the cell cycle

To determine the localization of CENP-C, CENP-T and CENP-W throughout the cell cycle, we added demembranated sperm nuclei to a CSF extract that was then cycled through interphase and subsequent mitosis. Aliquots of the assembly mixture were taken at different times and chromatin was spun onto coverslips to be analysed by immunoflourescence. We found that CENP-C is recruited to centromeres labeled by CENP-A already in the single chromatid chromosomes assembled in CSF and it is maintained there at all timepoints (Figure R2A). Previous reports have shown that, unlike CENP-A, CENP-C is not present in the sperm nuclei (Milks 2009) and that CENP-C most likely recognizes and binds directly to CENP-A nucleosomes (Carroll 2010; Guse 2011). CENP-T cannot be detected in CSF assembled chromosomes (Figure R2B). It targets the centromeres of interphase nuclei around 60 minutes after calcium addition, which corresponds to the timing of replication, and remains at centromeres until the next mitosis. CENP-W is targeted to the centromeres only in mitosis (Figure R2C). These observations suggest that CENP-C, CENP-T and CENP-W associate with centromeres at different times in the cell cycle.



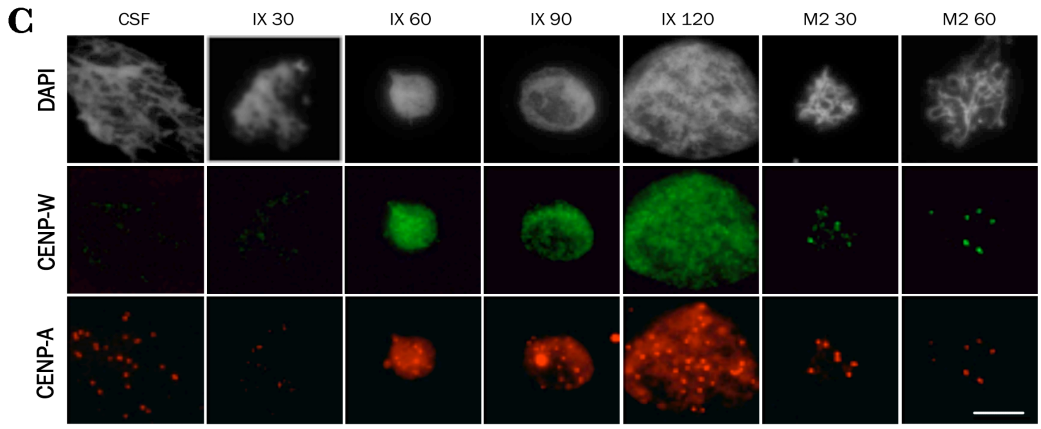


Figure R2. Localization of CENP-C, CENP-T and CENP-W through cell cycle

Time course of CENP-C (A), CENP-T (B) and CENP-W (C) localization in Xenopus egg extract. Sperm chromatin was incubated in CSF for 40 min and then cycled to interphase (IX) and second mitosis (M2). Nuclei and chromosomes obtained at the time points indicated were fixed and stained with antibodies against CENP-C, CENP-T and CENP-W (green), CENP-A (red) and DAPI (white). Scale bar is 10 μm.

The above results are particularly puzzling for CENP-T and CENP-W, since the two proteins have been reported to form a nucleosome-like particle and therefore one would expect to find them together on chromatin at all times. We reasoned that the CENP-W antibody used for immunofluorescent detection might recognize an epitope that is not exposed during interphase. To rule out this possibility, we analyzed by immunoblot chromatin assembled in CSF and then cycled to interphase and to mitosis (Figure R3A). Little CENP-W is present in interphase chromatin compared with the amount found in mitosis. indicating that CENP-W is not recruited until mitosis, unlike CENP-T. In the case of CENP-C, we also observed that a fraction of what is targeted in interphase is released in mitosis. Taking this result together with the observation of some staining outside centromeres by immunofluorescence (Figure R2A), it is likely that CENP-C incorporates transiently throughout chromatin in interphase, but only the population at centromeres remains bound in mitosis (Figure 3B).

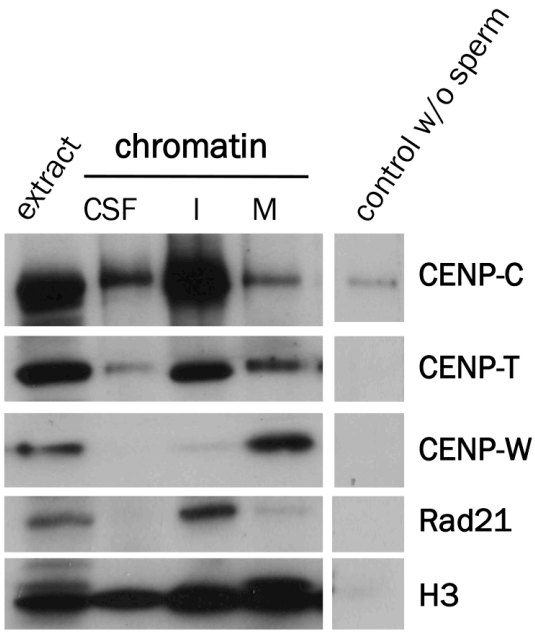


Figure R3. Centromeric targeting of CENP-C, CENP-T and CENP-W throughout the cell cycle

Immunoblot analysis of chromatin assembled in CSF, interphase (I) and mitotic (M) extracts and purified by centrifugation through a sucrose cushion (see Materials and Methods). Chromatin purified in the same way from a mock assembly reaction without sperm serves as control. Cohesin Rad21 is a marker for interphase and H3 is used as loading control marker.

5.1.3 Loading of new CENP-C in interphase follows the loading of new CENP-A and is independent of CENP-T

To further understand the requirements for the recruitment of CCAN proteins to chromatin, we first asked whether they are affected by the loading of new CENP-A, which takes place upon exit from CSF arrest in *Xenopus* egg extracts (Bernad et al., 2011). We first quantitated the amount of CENP-C present at centromeres in interphase nuclei compared the amount present in CSF chromosomes using the assay previously described for CENP-A (Figure M2; Bernad et al 2011), but measuring CENP-C staining instead of CENP-A staining. We observed an increase in CENP-C signal intensity, indicating that CENP-C also gets loaded in interphase (Figure R4A). Importantly, depletion of CENP-A from the extract, which does not affect the levels of soluble CENP-C (Figure R4B), abolishes both the loading of new CENP-A in interphase (Figure R4C) and the loading of more CENP-C (Figure R4D). We conclude that CENP-C binding in CSF extract saturates the CENP-A nucleosomes present in the sperm chromatin and loading of more CENP-C in interphase follows the incorporation of new CENP-A nucleosomes.

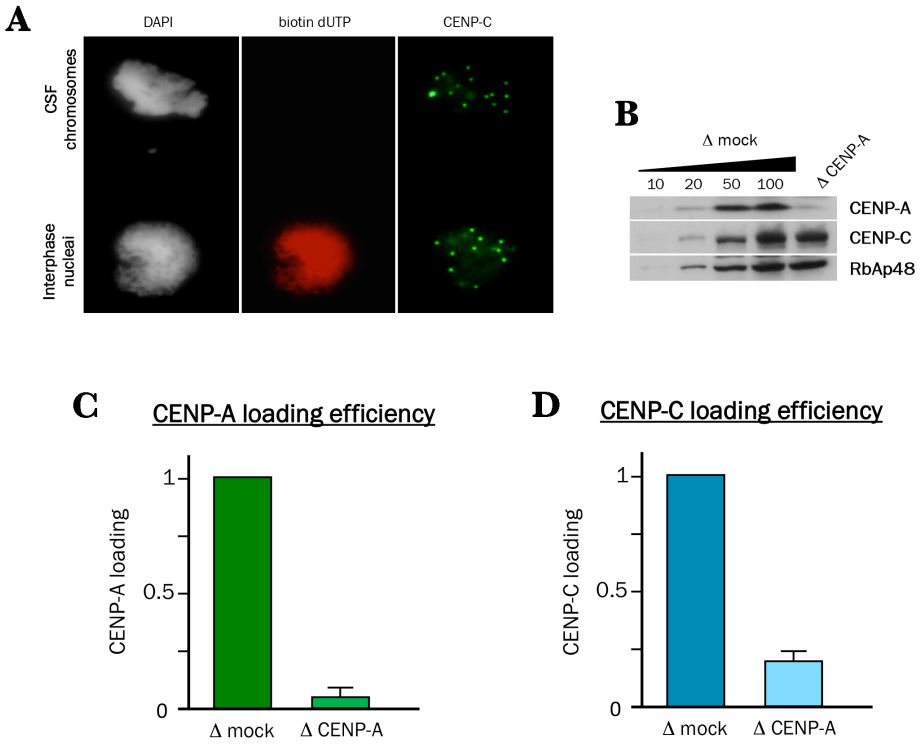


Figure R4. Loading of CENP-C depends on CENP-A loading

A) Representative images from CENP-C assembly assay showing CENP-C staining (red) of a mass of CSF chromosomes and an interphase nucleus, the latter already replicated as indicated by incorporation of biotin-dUTP (red).
B) Immunoblot analysis of increasing amounts of a mock-depleted extract (expressed as percentage of a 1.5-µl aliquot) and a 1.5-µl aliquot of a CENP-A depleted extract (Δ CENP-A). RbAp48 is used as a loading control.
C) Quantification of CENP-A loading efficiency in mock and CENP-A depleted extracts. More than 250 centromeres were measured for each condition. Error bars, SEM. n=3
D) Quantification of CENP-C in the same way as in C. Error bars, SEM. n=3

We also asked whether depletion of CENP-T or CENP-W had any effect on CENP-C binding to centromeres. Interphase nuclei and mitotic chromosomes were assembled in extracts with or without CENP-T and stained with antibodies against CENP-C and CENP-A. As shown in Figure R5, CENP-C can be detected at centromeres of interphase nuclei (A) and mitotic chromosomes lacking CENP-T. We conclude that the presence of CENP-T is not required for CENP-C recruitment, further supporting the possibility that CENP-C recognizes directly CENP-A.

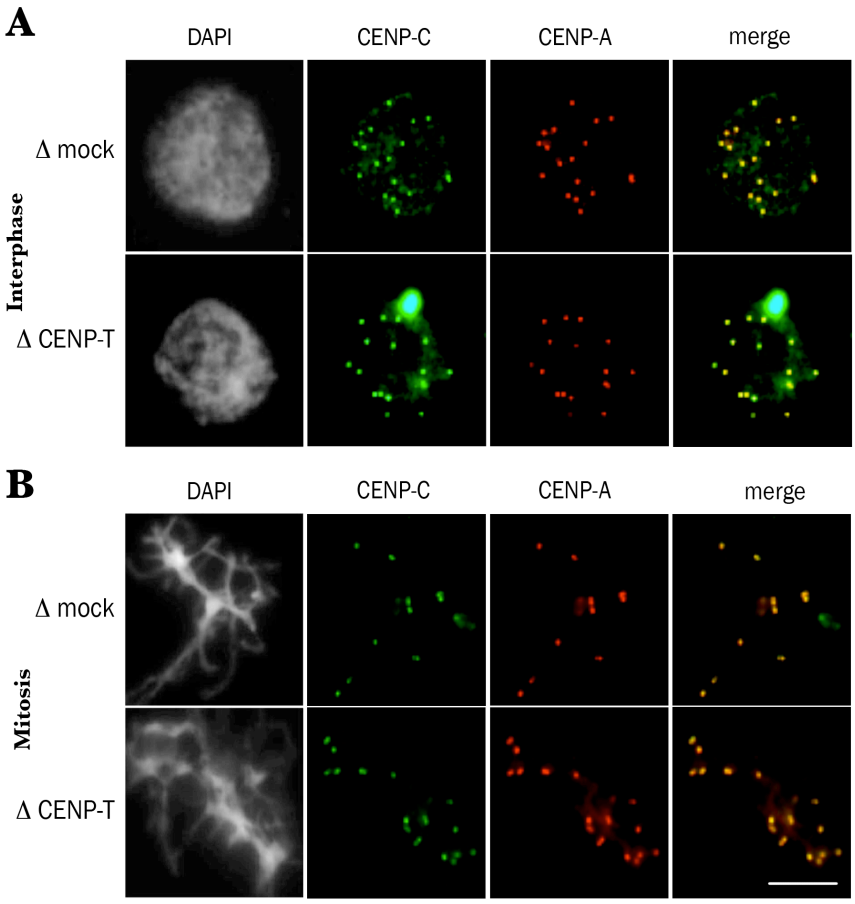


Figure R5. CENP-T depletion does not affect the recruitment of CENP-C.

Sperm chromatin was added to mock or CENP-T depleted (Δ) CSF extracts and cycled to interphase (A) and the following mitosis (B). Nuclei and chromosomes were fixed and stained with the indicated CENP-C (green), CENP-A (red) and DAPI (white). Scale bar is 10 μm.

When we did the same experiment in CSF extracts mock depleted or depleted of CENP-W, a clear decrease in the amount of CENP-C at centromeres could be observed (Figure R6, top and middle panels, and data not shown). However, this not due to a formal requirement of CENP-W for CENP-C recruitment, but to the fact that CENP-W depletion reduces amount of CENP-C present in the soluble extracts. When normal levels of CENP-C are restored in the CENP-W depleted extracts by addition of myc-tagged CENP-C it localizes to centromeres in the absence of CENP-W (R6, lower panel).

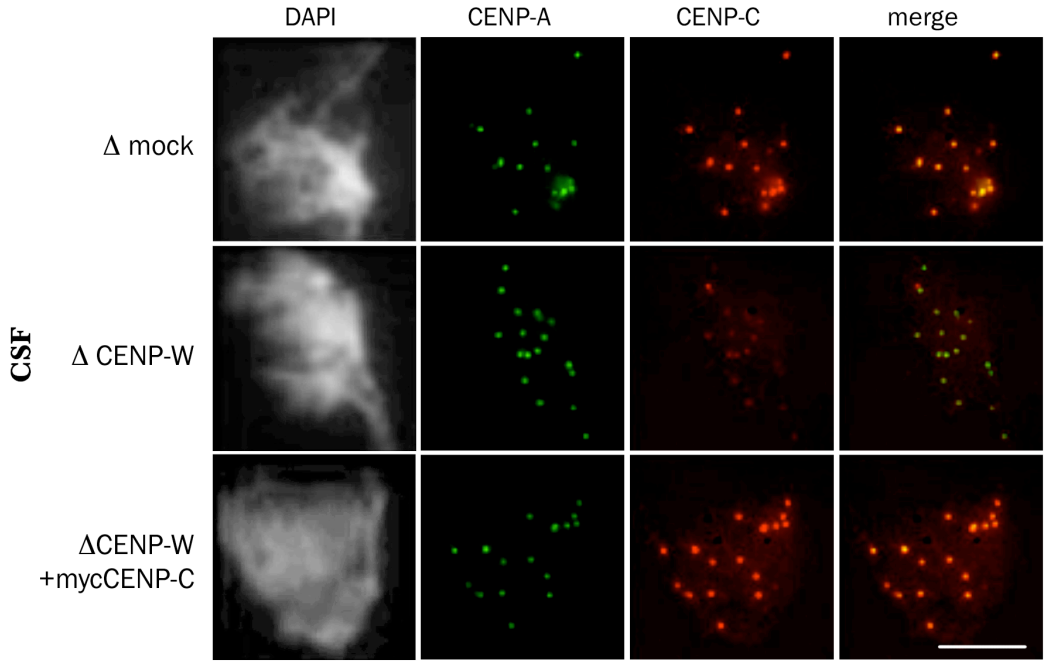


Figure R6. CENP-W depletion does not affect targeting of CENP-C in CSF.

Chromosomes assembled from sperm chromatin in CSF extracts mock-depleted, depleted of CENP-W and depleted of CENP-W but containing normal levels of CENP-C by addition of mycCENP-C were fixed and stained for CENP-C (green) and CENP-A (red). DAPI appears in white. Scale bar is 10 μm.

5.1.4 Loading of CENP-T in interphase is independent of DNA replication and de novo CENP-A deposition

Since CENP-T is recruited to centromeres during DNA replication, an obvious question is whether replication is a requirement for this recruitment. To test this possibility, we assembled nuclei in extracts containing the DNA polymerase inhibitor aphidicolin. Under this condition, no incorporation of biotinylated nucleotides can be observed but CENP-T is still found at centromeres (Figure R7A). Thus, CENP-T incorporation is independent of DNA replication and therefore of dilution of CENP-A with H3 nucleosomes. Next we tested a requirement for CENP-A deposition. We had previously shown that this deposition, despite taking place in interphase, requires previous passage though mitosis (Bernad et al., 2011). Thus, nuclei assembled by addition of sperm chromatin directly to interphase extract are not able to load new CENP-A. We found that CENP-T is properly targeted to centromeres also in this condition (Figure R7B).

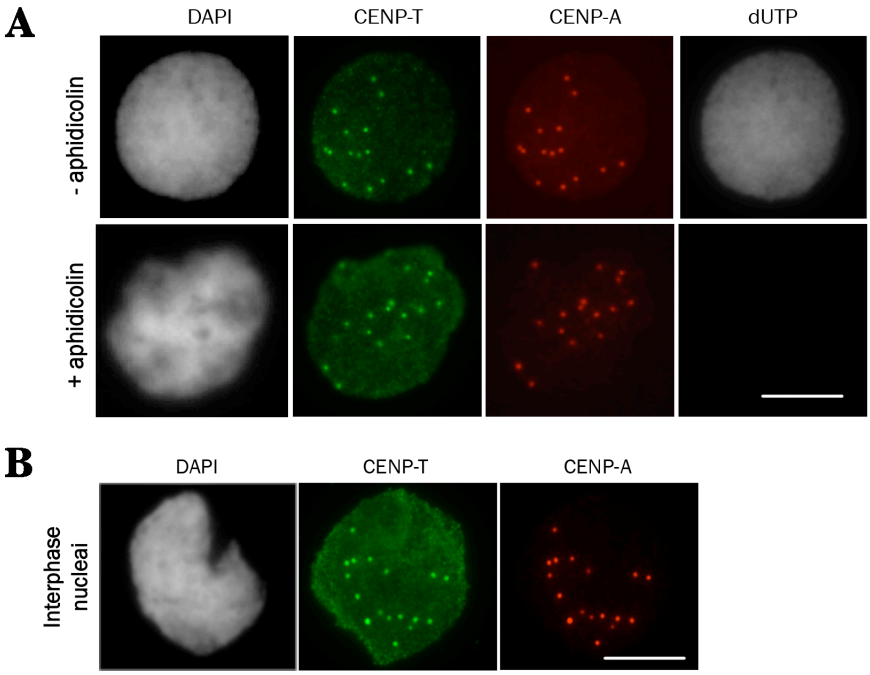


Figure R7. CENP-T targeting in interphase does not require DNA replication or CENP-A deposition.

A) Interphase nuclei assembled in extracts with and without treatment with aphidicolin to abolish DNA replication were fixed and stained with CENP-T (green), CENP-A (red) and DAPI. The absence of incorporation of biotinylated dUTP confirms the lack of DNA replication in the presence of aphidicolin.

B) CENP-T can localize to centromeres independently of new CENP-A loading. Images of sperm nuclei assembled in interphase and stained for CENP-T and CENP-A. Scale bar is 10 μm.

We also found that CENP-T localization in interphase is decreased but not abolished in CENP-C depleted extracts (Figure R8A), but it is abolished in mitosis (Figure R8B). We speculate that CENP-C stabilizes the binding of CENP-T to centromeric chromatin and this stabilization is particularly important during mitosis, when the complete kinetochore is assembled and functional.

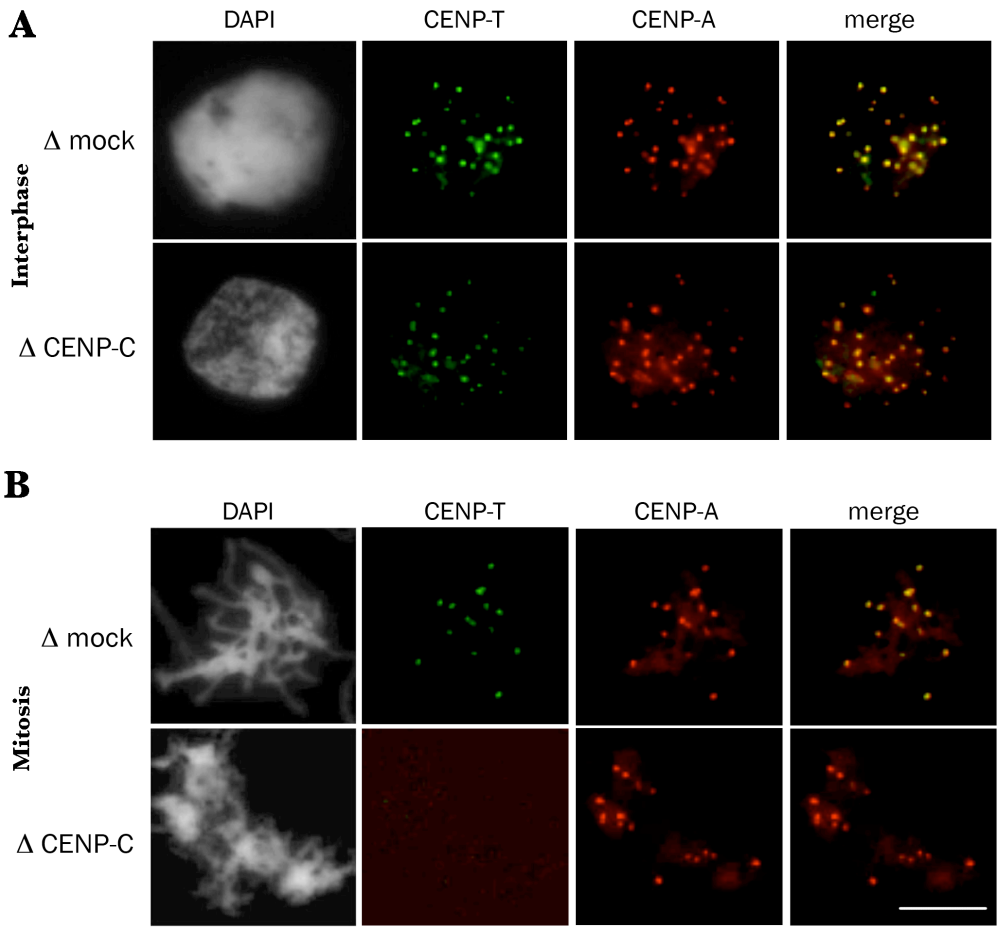


Figure R8. CENP-C depletion effects CENP-T maintenance at centromeres in mitosis.

Sperm chromatin was incubated in mock- or CENP-C-depleted CSF extracts and cycled to interphase and subsequent mitosis. Interphase nuclei (A) and mitotic chromosomes (B) were fixed and with CENP-T (green) and CENP-A (red). DNA was counterstained with DAPI (white). Scale bar is 10 μm.

Immunoblot analysis of chromatin fractions also shows some decrease of CENP-T on interphase chromatin assembled in CENP-C depleted extracts but the reduction is much more dramatic in mitotic chromatin (Figure R9, compare lanes 3-4 with 6-7). Finally, CENP-T does not strictly require CENP-W for centromeric targeting, although a decrease of CENP-T at centromeres under this condition can be observed both by immunoblot (Figure R9, lanes 12-13) and by immunofluorescence (Figure R10). This result is surprising given that CENP-W depletion partially depletes CENP-C. It is possible that a low amount of CENP-C is sufficient for CENP-T stabilization in mitosis.

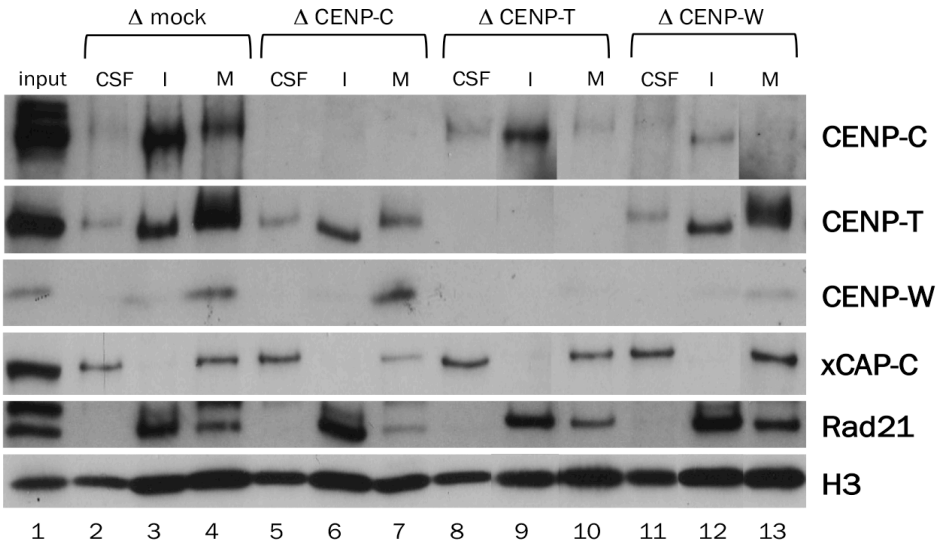


Figure R9. Immunoblot analysis of chromatin assembled after depletion of CCAN proteins.

Immunoblot analysis of chromatin assembled in mock, CENP-C, CENP-T and CENP-W depleted CSF extracts and cycled to interphase (I) and subsequent mitosis (M). Respective time points are indicated above each lane as well as depletion conditions. Rad21, a cohesin subunit, targets interphase nuclei and partially dissociates in mitosis. xCAP-C, a condensin subunit, is present in the condensed chromosomes both in CSF and mitosis. H3 serves as loading control.

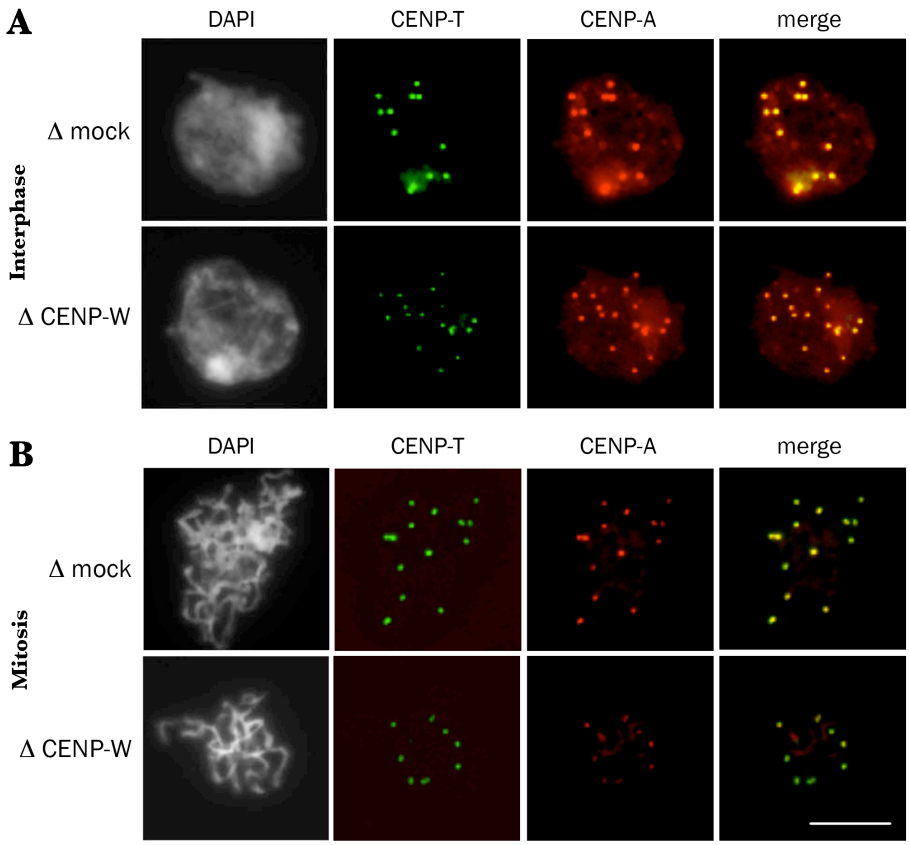


Figure R10. CENP-T is present on centromeric chromatin in the absence of CENP-W.

Sperm chromatin was incubated in mock- or CENP-W-depleted CSF extracts and cycled to interphase and subsequent mitosis. Interphase nuclei (A) and mitotic chromosomes (B) were fixed and stained with antibodies recognizing CENP-T (green) and CENP-A (red). DNA was counterstained with DAPI (white). Scale bar is 10 μm.

5.1.5 Requirements for CENP-W loading onto centromeres

As for CENP-T, binding of CENP-W to centromeric chromatin in mitosis does not require deposition of new CENP-A in the preceding interphase (Figure R11A). However, it is dependent on CENP-C (Figure R11B) and on CENP-T (Figure R11C). Intriguingly, we found some discrepancies between our observations by immunofluorescence and by immunoblot analysis. For example, CENP-W can be detected on mitotic chromatin assembled in the CENP-C depleted extract by immunoblot (Figure R9, lane 7) but not by immunofluorescence. At present we do not understand the reason for this discrepancy.

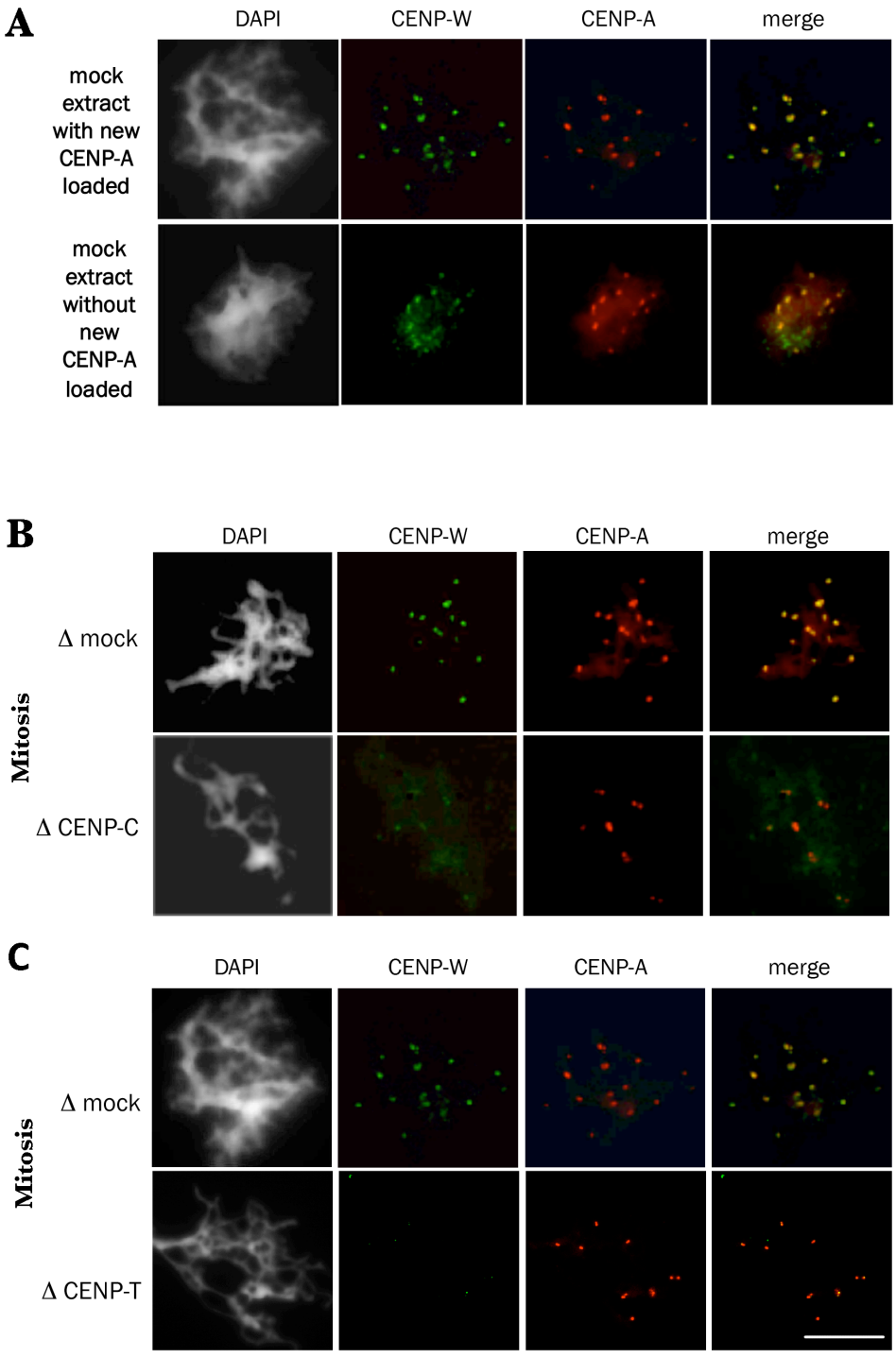


Figure R11. Localization of CENP-W does not depend on CENP-A loading in preceding interphase but requires CENP-C and CENP-T.

A) Sperm chromatin was incubated in CSF extracts (top) and interphase extracts (bottom) and cycled to the next mitosis. Only in the first condition there is loading of new CENP-A in interphase. Mitotic chromosomes were fixed and stained for CENP-W (green) and CENP-A (red). DNA was counterstained with DAPI (white).

B) Sperm chromatin incubated in mock, CENP-C or CENP-T depleted CSF extracts was cycled to interphase and subsequent mitosis. Chromosomes were fixed and stained for CENP-T (green), CENP-A (red) and DAPI (white). Scale bar is 10 μm.

5.1.6 Role of CENP-C, CENP-T and CENP-W in kinetochore assembly

It has been postulated that the CCAN protein network connects centromeric chromatin to the outer kinetochore and thereby has an essential role in chromosome segregation. To confirm that this role is conserved in our cell-free system, we decided to look at the localization of the KMN network proteins. This network, constituted by the Knl1 complex, the Mis12 complex and the Ndc80 complex, is part of the protein architecture within kinetochores that links centromeric DNA to the plus ends of spindle microtubules. We assembled mitotic chromosomes in extracts depleted from either CENP-C or CENP-T or CENP-W and looked at the recruitment of Ndc80 to the centromeres, marked by CENP-A (Figure R12). We observed that the absence of any of the three proteins compromises the correct targeting of Ndc80 to centromeres.

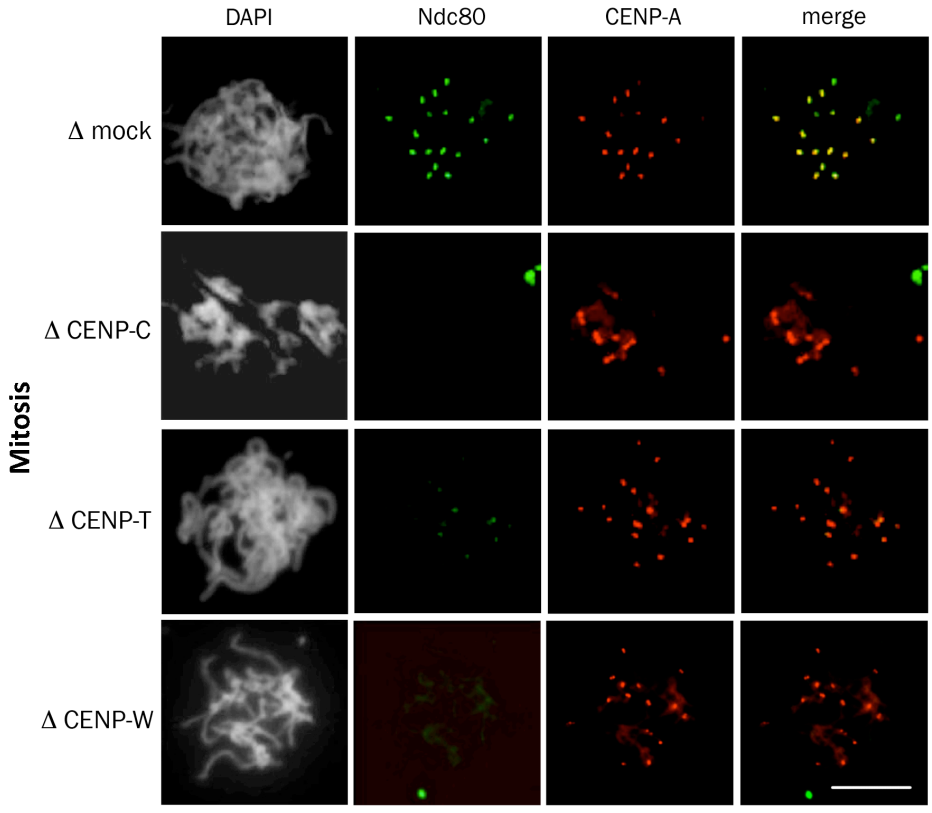


Figure R12. Depletion of either CENP-C or CENP-T or CENP-W abolishes proper targeting of kinetochore protein Ndc80.

Mitotic chromosomes were assembled in extracts depleted of CENP-C, CENP-T or CENP-W, fixed and stained with antibodies against Ndc80 (green) and CENP-A (red). DNA was counterstained with DAPI (white). Scale bar is 10 μm.

Recruitment of the other major kinetochore component, Mis12, was also clearly affected in the absence of CENP-T (Figure R13). Unfortunately, we had very limited amount of the Mis12 antibody and therefore we could not performed similar stainings in the CENP-C or CENP-W depleted chromosomes.

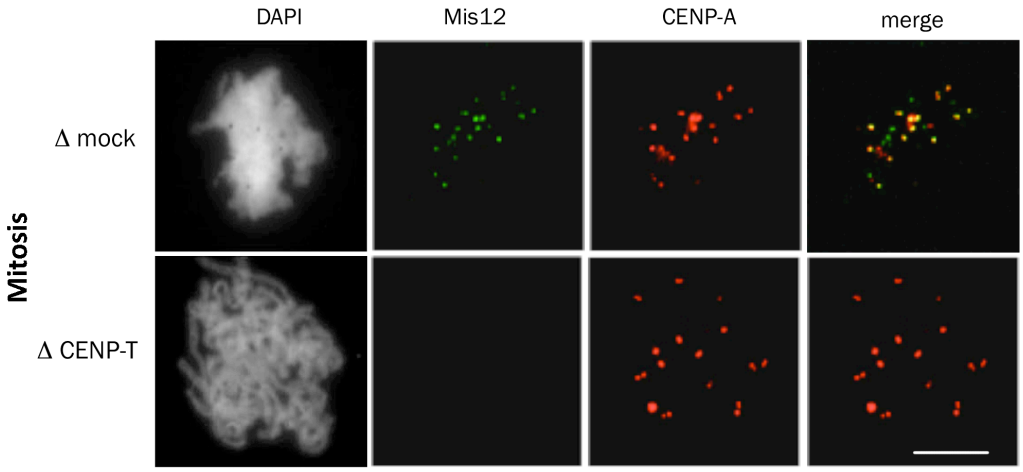


Figure R13. Depletion of CENP-T impairs the recruitment of kinetochore protein Mis12.

Sperm chromatin was incubated in mock- or CENP-T-depleted CSF extracts and cycled through interphase to the next mitosis. Chromosomes were then fixed and stained for Mis12 (green) and CENP-A (red). DNA was counterstained with DAPI (white). Scale bar, 10 μm.

We also found that adding back CENP-T to the CENP-T depleted extracts did not restore Ndc80 targeting (Figure R14A). CENP-T depletion co-depletes a large fraction of CENP-W (Figure R14B, lane). When mycCENP-W is added back together with CENP-T to the CENP-T depleted extract, Ndc80 can be detected at the centromeres of mitotic chromosomes (Figure R14A, bottom). These data indicate that the three CCAN components CENP-C, CENP-T and CENP-W need to be present for proper kinetochore assembly in mitosis.

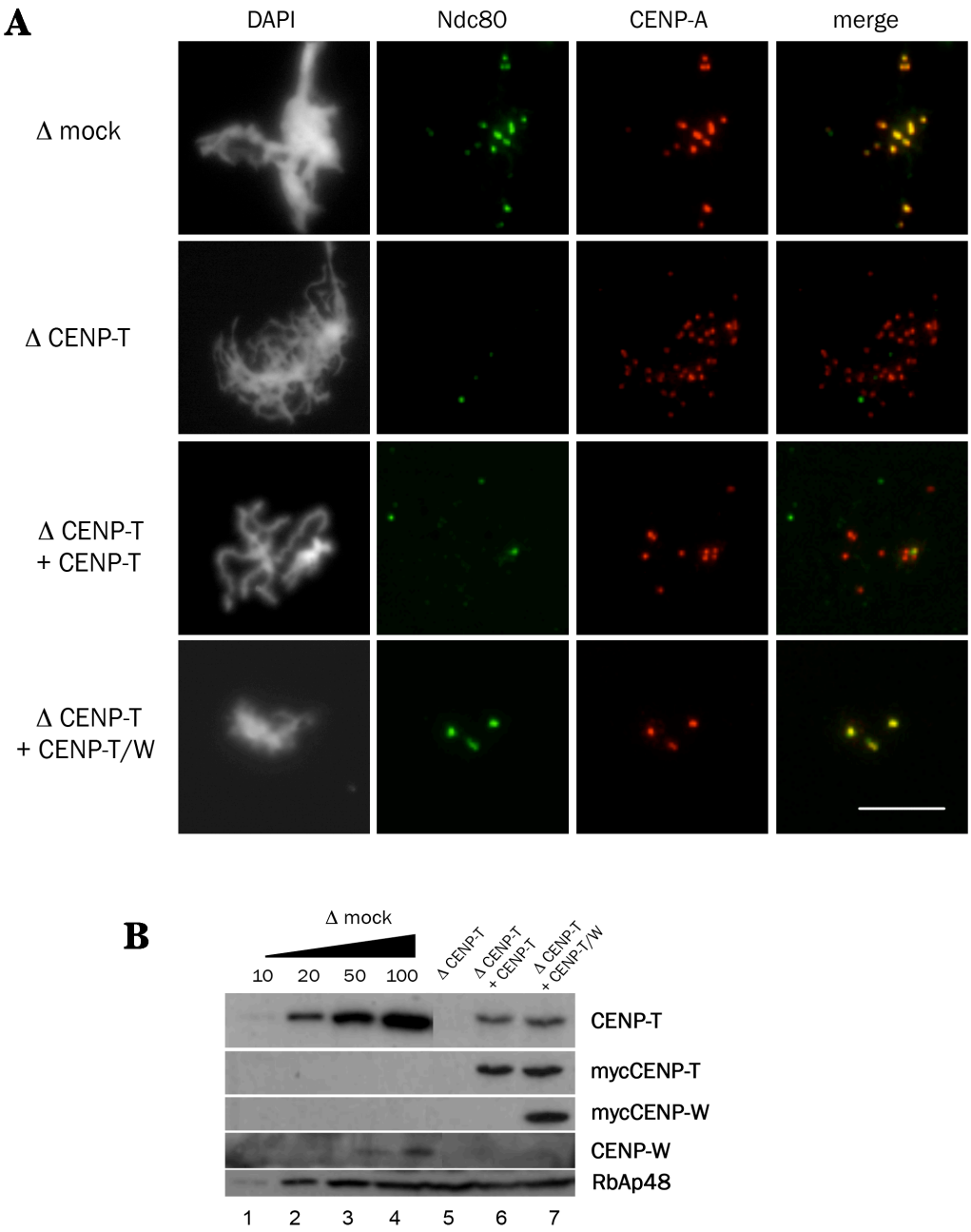


Figure R14. Proper Ndc80 localization requires both CENP-T and CENP-W.

A) Mitotic chromosomes were assembled in mock-depleted extracts or extracts depleted of CENP-T and reconstituted with buffer, CENP-T alone of both CENP-T and CENP-W. The add-back proteins were in vitro translated in rabbit reticulocyte lysates). Chromosomes were fixed and stained with antibodies against Ndc80 (green) and CENP-A (red). DNA was counterstained with DAPI (white).

B) Immunoblot analysis of increasing amounts of a mock-depleted extract (expressed as percentage of a 1.5-µl aliquot) and a 1.5-µl aliquot of a CENP-T depleted extract (Δ CENP-T) and CENP-T depleted extract reconstituted with only CENP-T or with both CENP-T and CENP-W proteins in vitro translated in reticulocyte lysates. RbAp48 is used as a loading control.

5.1.7 Role of CENP-C, CENP-T and CENP-W in the loading of new CENP-A

Previously, we developed an *in vitro* assay to measure this incorporation of new CENP-A in chromosomes assembled in *Xenopus* egg extracts (Bernad et al, 2011). In this assay, chromosomes are assembled in CSF extract from sperm chromatin and then part of this mixture is converted to interphase nuclei. CSF chromosomes and interphase nuclei are then spun down together over coverslips and processed for immunofluorescence with a CENP-A specific antibody. Pairs of CSF chromosomes and interphase nuclei are imaged (see an example in Figure R15), and the centromeric CENP-A signals measured to assess the average difference in intensity between the CSF and interphase centromeres within each imaged pair. Further details of the assay are given in Material and Methods, section 7.6. Using this assay, we assessed the role of CENP-C, CENP-T and CENP-W in CENP-A deposition.

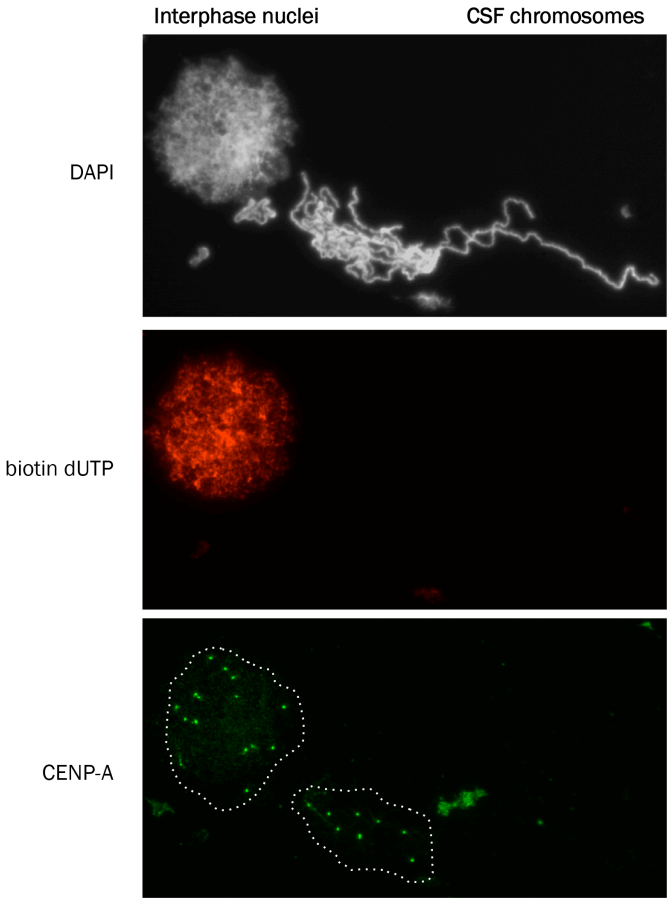


Figure R15. An assay to quantitate CENP-A incorporation at centromeres in the *Xenopus* egg cell-free system

Example of the images analyzed for a CENP-A assembly assay. The interphase nucleus on the left has incorporated biotin-dUTP during replication. The CSF chromosomes on the right have not. The centromeric CENP-A signals (green) are then measured to assess the average difference in intensity between the centromeres within each pair. Interphase centromeres are marked on the left and CSF ones are circled on the right by dotted lines.

CENP-C is essential for the loading of new CENP-A

Depletion of CENP-C from the soluble extract to more than 90% (Figure R16A, lane 7) does not affect CENP-A levels (data not shown). However, loading of new CENP-A is severely impaired (around 20% of the loading in the mock depleted extract; Figure R16B). When mRNA of a *myc*-tagged version of CENP-C is added back to the depleted extract, the corresponding mycCENP-C is synthesized (Figure R16A, lane 8) and is recruited to centromeres (Figure R16C). Under this condition, CENP-A loading is restored to normal levels (Figure R16B). Thus, CENP-C is essential for the assembly of new CENP-A.

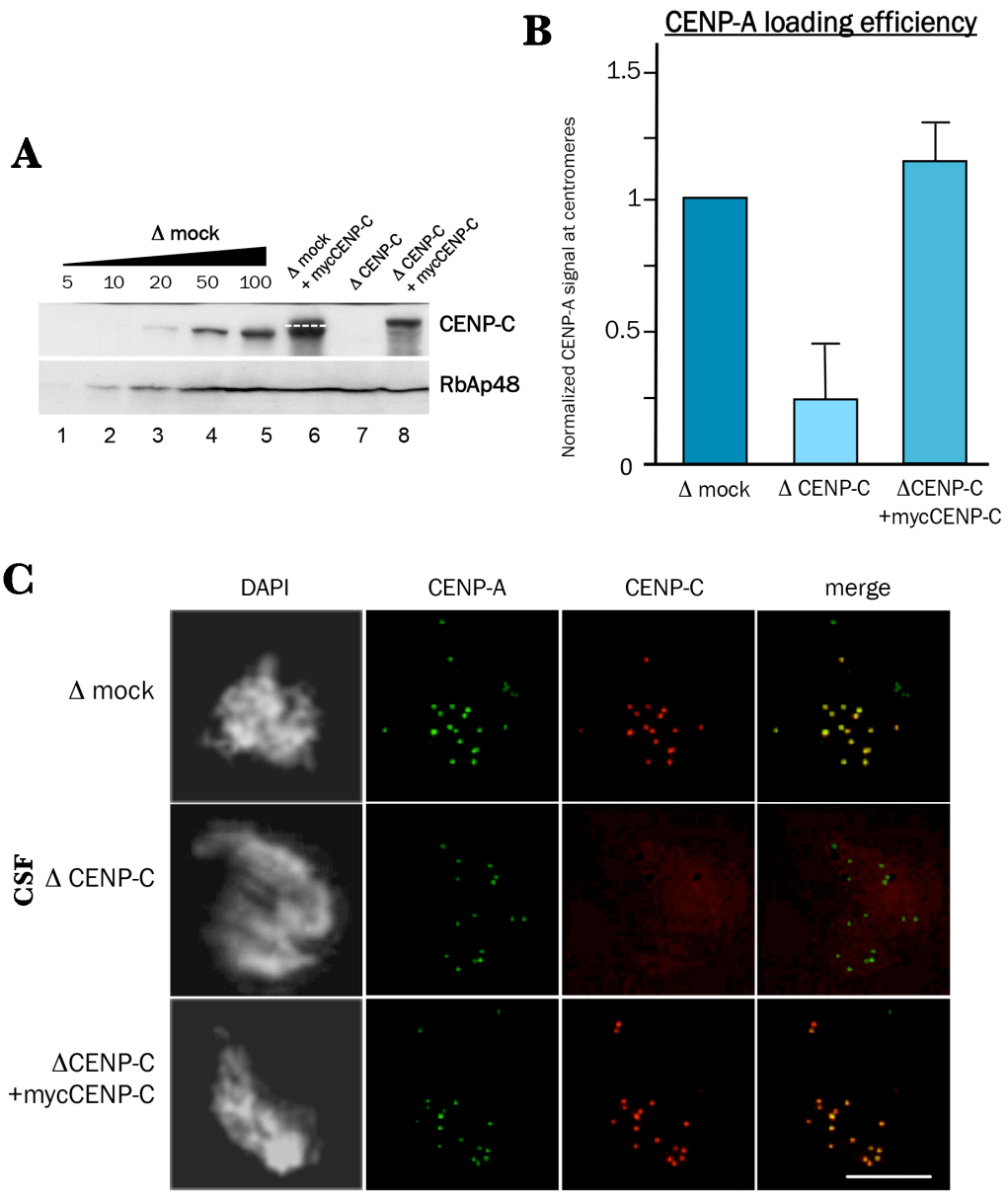


Figure R16. CENP-C is essential for CENP-A loading.

Figure R16. CENP-C is essential for CENP-A loading (continued).

A) Immunoblot analysis of increasing amounts of a mock-depleted CSF extract (Δ mock, expressed as percentage of a 1.5-µl aliquot) and 1.5-µls aliquot of a CENP-C depleted CSF extract (Δ CENP-C) without and with myc CENP-C mRNA added. In line 6 above the white line is myc CENP-C fraction while the one below is of endogenous CENP-C. RbAp48 is used as a loading control.
B) Bar graph representing CENP-A loading efficiency in nuclei assembled in the indicated extracts. More than 250 centromeres were quantitated per condition and experiment. Error bars, SEM. n≥5
C) CSF chromosomes assembled in the experimental conditions described in A) were fixed and stained for CENP-C (red) and CENP-A (green). DNA was counterstained with DAPI (white). Scale bar is 10um.

CENP-T and CENP-W do not have a role in loading of new CENP-A

CENP-W protein is difficult to visualize by western blot. Thus, to better quantitate the extent of its depletion, we added a myc- tagged version of CENP-W to the extract before depletion. Figure R17B shows a reduction of at least 90% of the exogenous *myc*CENP-W and a bit less for the endogenous CENP-W after the incubation with antibody beads. De novo loading of CENP-A was severely impaired in the chromatin assembled in the CENP-W depleted extract (Figure R17A). Moreover, the add-back of *myc*CENP-W to the extract did not rescue this defect (Figure R17A). Since we had previously noticed that CENP-W partially depletes CENP-C protein (Figure R1B, lane 7) and CENP-C is essential for CENP-A loading, we reasoned that the limited amounts of CENP-C and not the lack of CENP-W *per se* could be responsible for the observed defect in CENP-A loading in the CENP-W depleted extracts. Indeed, when *myc*CENP-C is added to the CENP-W depleted extract, CENP-A loading is restored to normal levels even though CENP-W is not present in this extract (Figure R17A). Consistent with this finding, depletion of more than 90% of CENP-T does not affect CENP-A assembly (Figure R18). Taken all together, these results indicate that CENP-C is essential for CENP-A deposition in early interphase whereas CENP-T and CENP-W are dispensable.

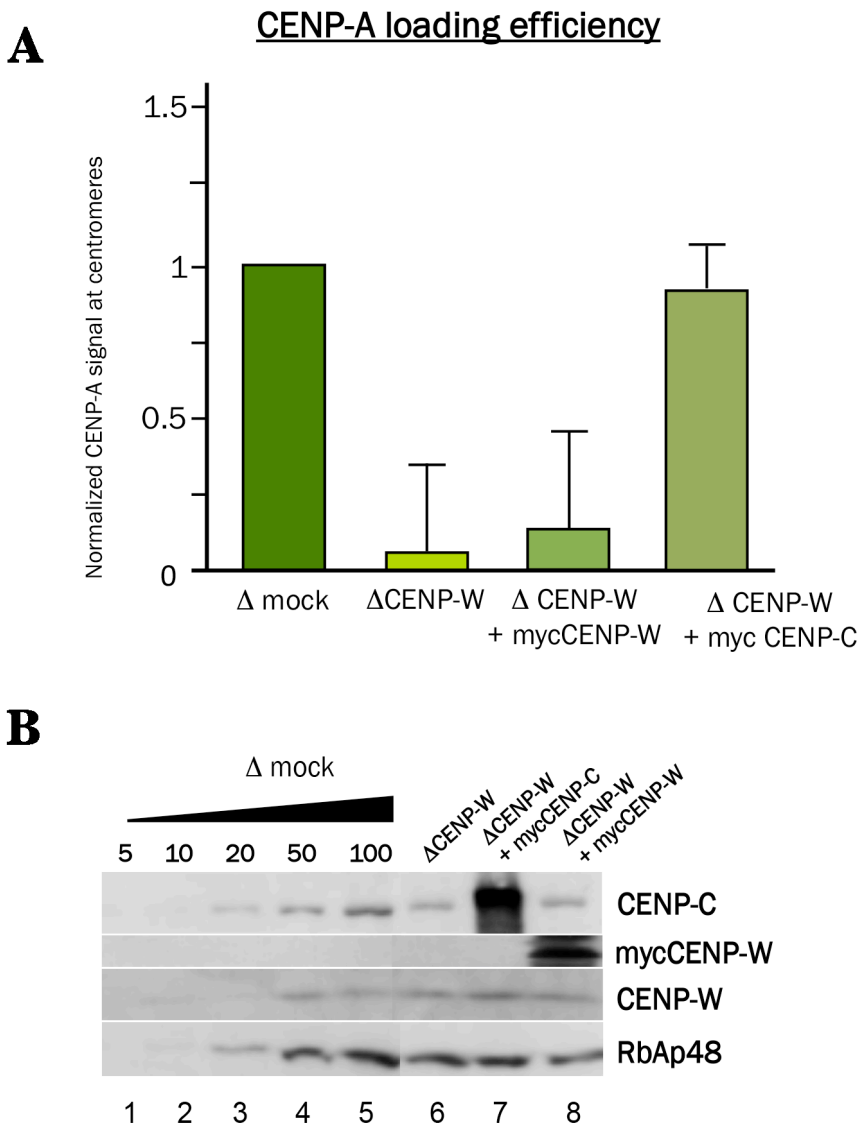


Figure R17. CENP-W depletion affects CENP-A loading but it is not essential

A) Bar graph representing CENP-A loading efficiency in nuclei assembled in the indicated extracts. More than 250 centromeres were quantitated per condition and experiment. Error bars, SEM. n≥5

B) Immunoblot analysis of increasing amounts of a mock-depleted CSF extract (Δ mock, expressed as percentage of a 1.5-µl aliquot) and 1.5-µls aliquot of a CENP-W depleted CSF extract (Δ CENP-W) reconstituted with buffer, myc CENP-C mRNA or *in* vitro translated CENP-W protein. RbAp48 is used as a loading control.

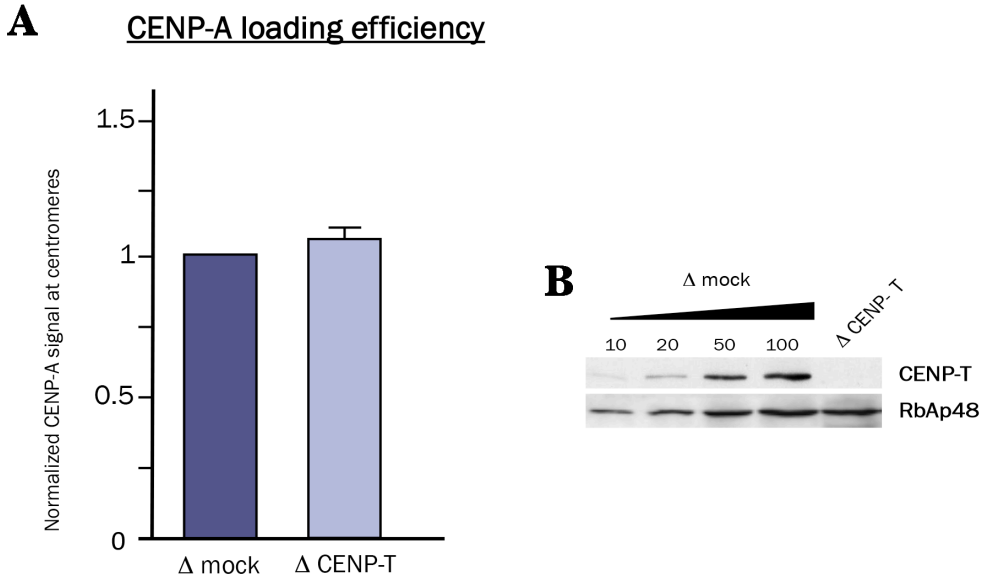


Figure R18. CENP-T is not essential for CENP-A loading.

A) Quantification of CENP-A loading efficiency in mock and CENP-T depleted extracts. More than 250 centromeres were measured for each condition. Error bars, SEM. n=5.

B) Immunoblot analysis of increasing amounts of a mock-depleted CSF extract (Δ mock, expressed as percentage of a 1.5-µl aliquot) and 1.5-µls aliquot of a CENP-T depleted CSF extract (Δ CENP-T). RbAp48 is used as a loading control.

5.1.8 CENP-C orchestrates centromere targeting of multiple factors

CENP-C may target M18BP1 to centromeres

Given the direct interaction between CENP-C and CENP-A, we speculated that the essential role of CENP-C in CENP-A assembly could derive from a role of CENP-C in targeting factors required for the loading process to centromeric chromatin. As described in the Introduction, the CENP-A specific chaperone HJURP and the Mis18 complex are both essential for CENP-A assembly. We previously showed that there is a HJURP homolog in *Xenopus* and this protein is stored in the egg extract associated with CENP-A (Bernad et al., 2011). The components of the Mis18 complex, Mis18a, Mis18b and M18BP1 are also present in *Xenopus* (Maddox et al., 2007; Moree et al., 2011; D. Foltz, personal communication). To test if CENP-C interacts with these proteins we first performed immunoprecipitation reactions from both CSF and interphase extracts with antibodies that we had previously raised against Mis18a and M18BP1 (Figure R19A). We could detect CENP-C in the M18BP1 immunoprecipitates from CSF extract, but not in those obtained

from interphase extract (Figure R19A, compare lane 6 and lane 12). Moreover, M18BP1 interacts with Mis18a only in interphase (Figure R19A, lane 9). Mis18a antibodies pull down CENP-C neither from CSF nor from interphase extracts. However, they pull down one of the two isoforms of M18BP1 as well as HJURP in interphase (Figure R19A, lane 11). Together, these cell-cycle specific interactions suggest that CENP-C may bring M18BP1 to centromeres already in CSF, as recently suggested by Moree et al (2011). Upon entry into interphase, M18BP1 brings Mis18 and this, in turn, recruits HJURP (Figure 19B). Further experiments are required to prove this model. Unfortunately, the antibodies against Mis18a and M18BP1 do not work well for immunofluorescence and we have been unable to detect a myc-tagged version of Mis18a at centromeres at any time.

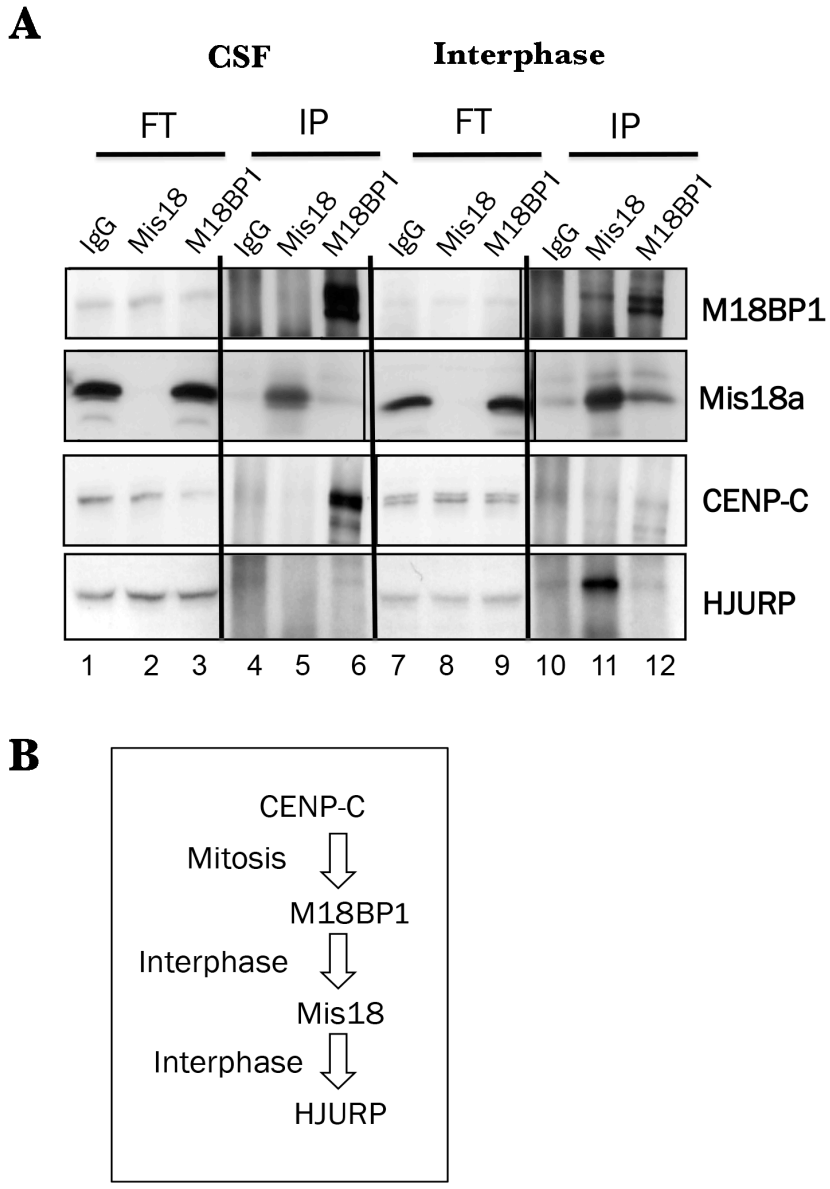


Figure R19. CENP-C interacts with M18BP1 in mitosis.

Figure R19. CENP-C interacts with M18BP1 in mitosis (continued).

A) Immunoblot analysis of immunoprecipitates (IP) obtained from *Xenopus* LSS extract with specific antibodies against Mis18 and M18BP1. Immunoprecipitation with non-immune rabbit IgG was used as control. Aliquots of the flow through (FT) from each reaction were also analyzed.
B) Model for the recruitment of HJURP by CENP-C through Mis18 and M18BP1.

What we could observe, however, is that recruitment of HJURP to centromeres in interphase and mitosis is strongly reduced upon the depletion of CENP-C (Figure R20A, B). This defect is not due to variation in HJURP protein levels upon CENP-C depletion (Figure R21B) and is consistent with the pathway proposed above.

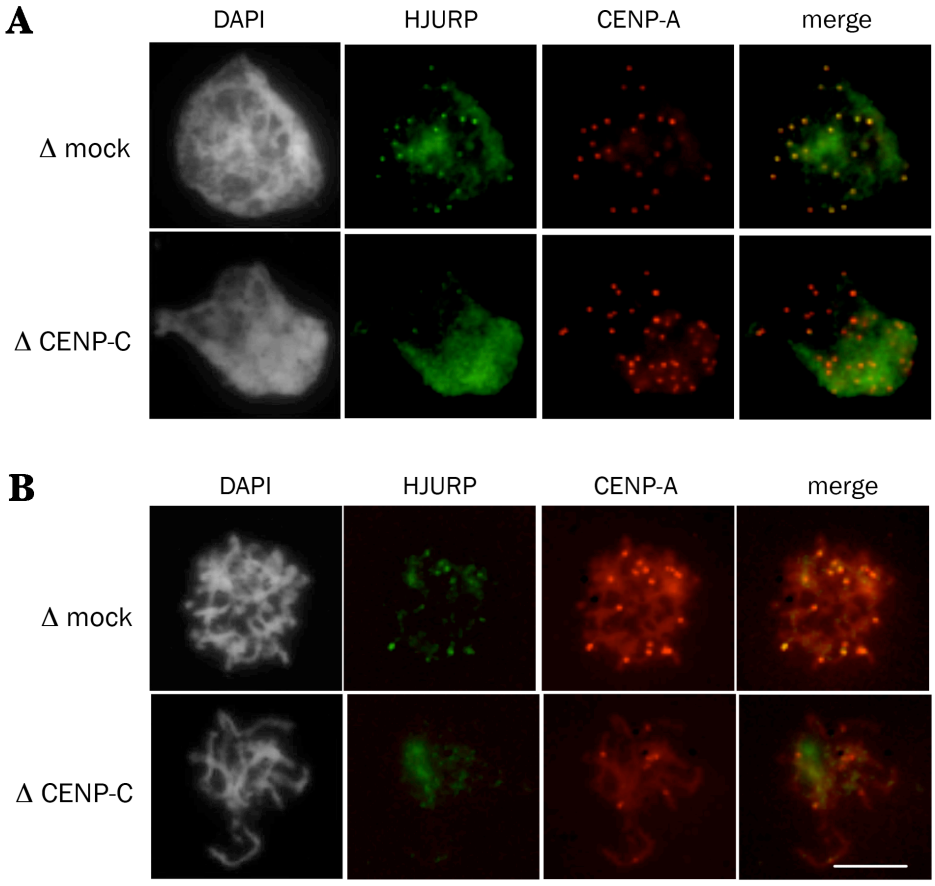


Figure R20. CENP-C is necessary for proper recruitment of CENP-A-specific chaperone HJURP to centromeres

A) Chromosomes assembled in CSF control extract and extract depleted of CENP-C protein were cycled to interphase, fixed and stained for HJURP (green), CENP-A (red) and DAPI. Scale bar is 10um.
B) CSF chromosomes assembled in the experimental conditions described in A) were cycled to mitosis, fixed and stained for HJURP (green) and CENP-A (red). DNA was counterstained with DAPI (white). Scale bar is 10um.

CENP-C interacts directly with HJURP

Intriguingly, we found that CENP-C interacts with HJURP in CSF and interphase extracts (Figure R21A). What is more, CENP-C is largely co-depleted upon HJURP depletion (Figure R21B). Under this condition, little CENP-C can be detected at centromeres (Figure R21C). Adding back *myc*CENPC mRNA to HJURP depleted extracts, however, restores CENP-C targeting. This results supports once again that CENP-C binds directly to CENP-A. It is important to note that depletion of CENP-C does not affect the levels of soluble HJURP. Thus, the effect of CENP-C depletion on CENP-A incorporation is not due to the removal of HJURP from the extract but to impairment of its centromeric recruitment.

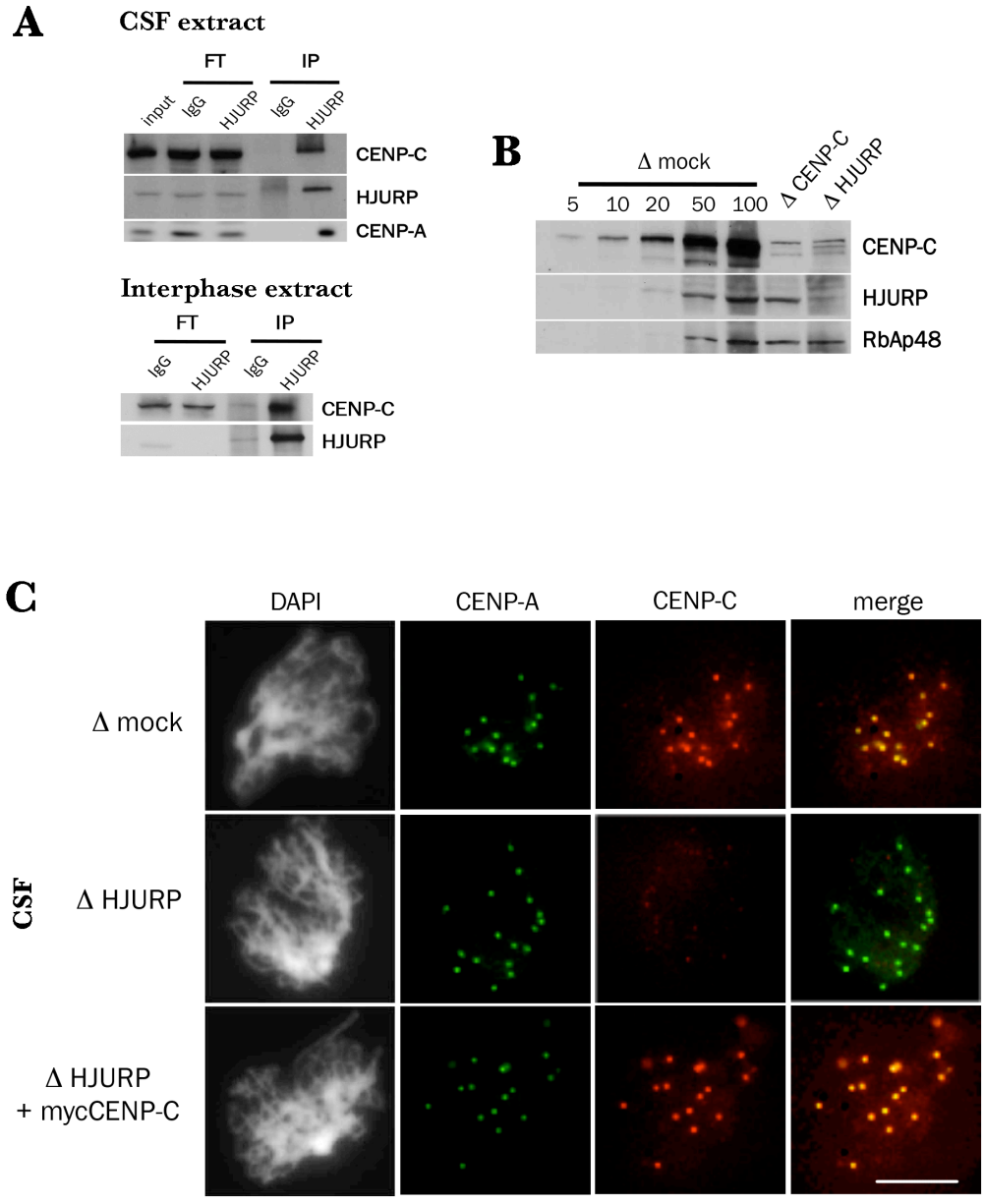


Figure R21. CENP-C interacts with HJURP.

Figure R21. CENP-C interacts with HJURP (continued).

A) Immunoblot analysis of immunoprecipitates (IP) obtained from CSF and interphase extracts with antibodies against HJURP. As control, immunoprecipitation with non-immune rabbit IgG was used. Aliquots of flow-through (FT) reactions and the input extract were also analyzed.

B) Immunoblot analysis of 1.5-µl aliquots of extracts depleted with specific antibodies (Δ) alongside increasing amounts of a mock-depleted extract (expressed as percentage of a 1.5-µl aliquot). RbAp48 is used as a loading control.

C) Chromosomes assembled in CSF extract undepleted and depleted from HJURP protein and reconstituted with buffer or mycCENP-C mRNA were fixed and stained for CENP-C (red), CENP-A (green) and with DAPI (white). Scale bar is 10um.

CENP-C associates with the chromatin remodeler FACT

In *Xenopus laevis,* the chromatin remodeler FACT is described as DNA unwinding factor (DUF) and has a role in DNA replication (Okuhara et al., 1999). It consists of two subunits, FACTp140 (xDUF140/Spt16) and FACTp87 (xDUF87/SSRP1). We had raised antibodies against both FACT components but only the one against FACTp140 is reliable and works well for immunoblot, immunoprecipitation, immunofluorescence and depletion. A clear interaction between CENP-C and FACT proteins can be detected by coimmunoprecipitation from the soluble egg extracts (Figure R22). As we will describe in the next section, FACT localizes at centromeres in mitosis and has an essential role in CENP-A deposition in *Xenopus*.

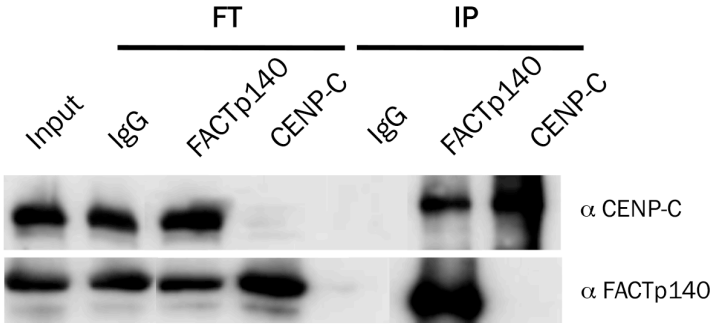


Figure R22. CENP-C interacts with FACT

Immunoprecipitates (IP) from CSF extracts with antibodies against FACT subunit p140 and CENP-C were analyzed by immunoblot. Immunoprecipitation with non-immune rabbit IgG was performed as control. Input extract and flow-through (FT) of the immunoprecipitates were analyzed as well.

CENP-C forms a complex with HJURP, FACT and CENP-W

Because we observed that CENP-C interacts with CENP-W, HJURP and FACT in the soluble egg extracts, we wondered if all these proteins form a single complex. To address this question, we first analyzed the fractions from a sucrose gradient shown in Figure R1C with antibodies against HJURP and FACTp140 (Figure R23). Most CENP-C appears in a fraction that corresponds to a sedimentation coefficient around 14-15S and in which CENP-W, FACT and HJURP can also be dectected. Additionally, when probed with CENP-A, we also observed a fraction of HJURP with CENP-A at around 11S. Interestingly, we also see FACTp140 in this fraction.

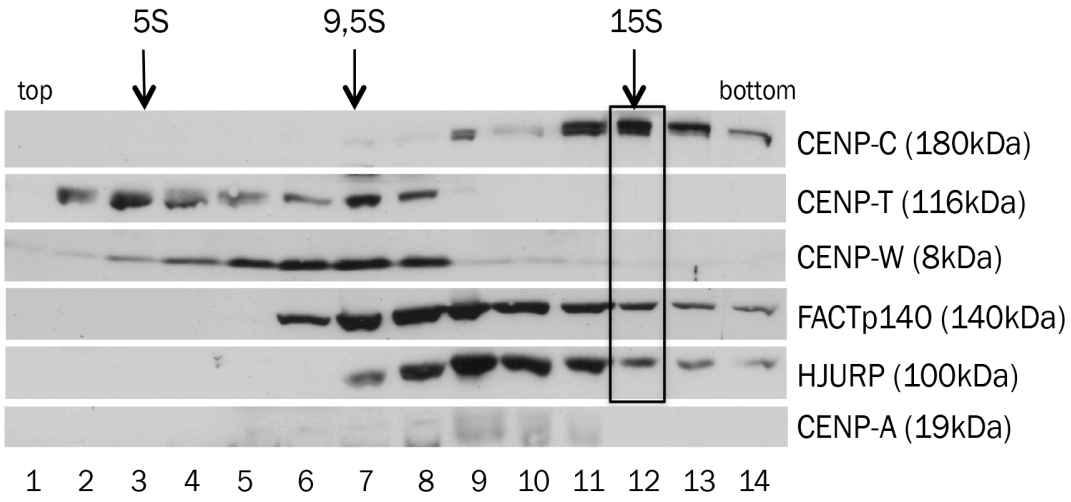


Figure R23. CENP-C associates with CENP-W, FACT and HJURP in the soluble extract.

Fractions collected from the fractionation of CSF HSS egg extract on a sucrose gradient, previously shown in Figure R1C, were now analyzed by immunoblot with FACTp140 and HJURP antibodies. Both proteins can be found in the 15S peak containing CENP-C.

Further proof of the existence of a complex containing the four proteins comes from the analyses of immunoprecipitation reactions from mock, CENP-W, HJURP and FACT depleted extracts with antibodies against each of these proteins. We found that the amount of CENP-C pulled down with all three antibodies was clearly reduced upon depletion of any of the three proteins (Figure R24), something unlikely to happen if CENP-C formed distinct complexes with CENP-W, FACT and HJURP.

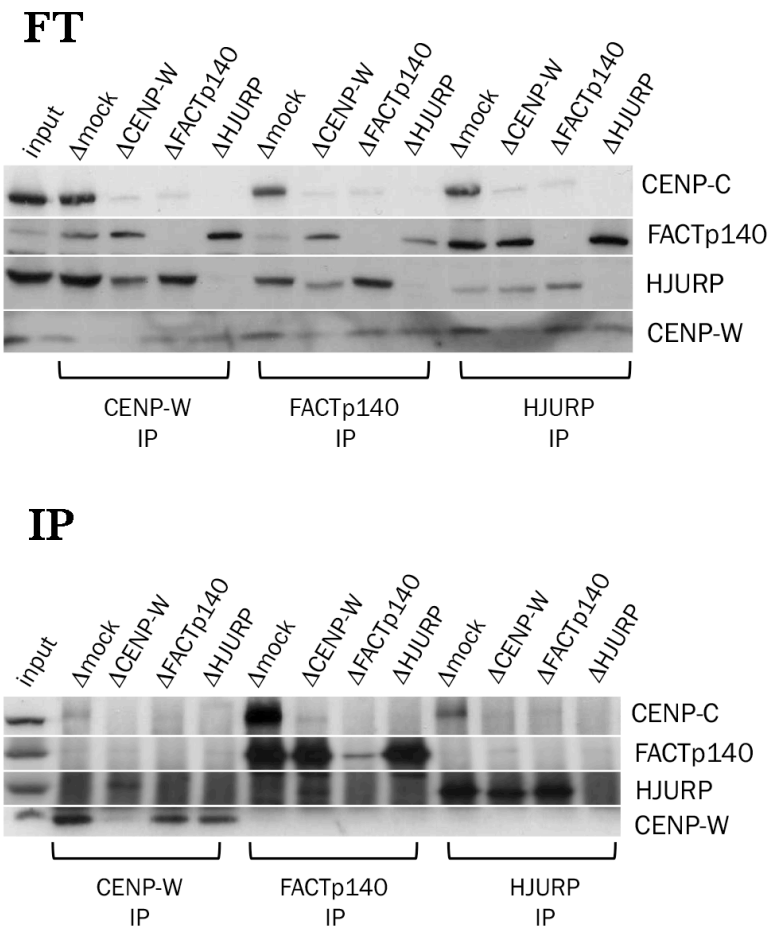


Figure R24. CENP-C forms a complex with CENP-W, FACT and HJURP
Immunoprecipitates (IP) from *Xenopus* HSS extracts mock-depleted and depleted from CENP-W, FACT and HJURP were obtained with antibodies against the same proteins. Aliquots of the flow through (FT) from each reaction and of the input extract were also analyzed.

The fact that we cannot detect FACT, CENP-W and HJURP in the immunoprecipitates of CENP-C or even in the immunoprecipitates obtained with antibodies against each of the three proteins (Figure R25A) probably reflects the very low abundance of CENP-C in the soluble egg extracts. For the same reason, depletion of CENP-C does not affect the levels of HJURP, FACT or CENP-W whereas depletion of any of these three proteins dramatically diminishes CENP-C levels (Figure R25B). Importantly, CENP-C depletion prevents centromeric targeting of CENP-W (Figure R11B), HJURP (Figure R20) and FACT (Figure R27, next section).

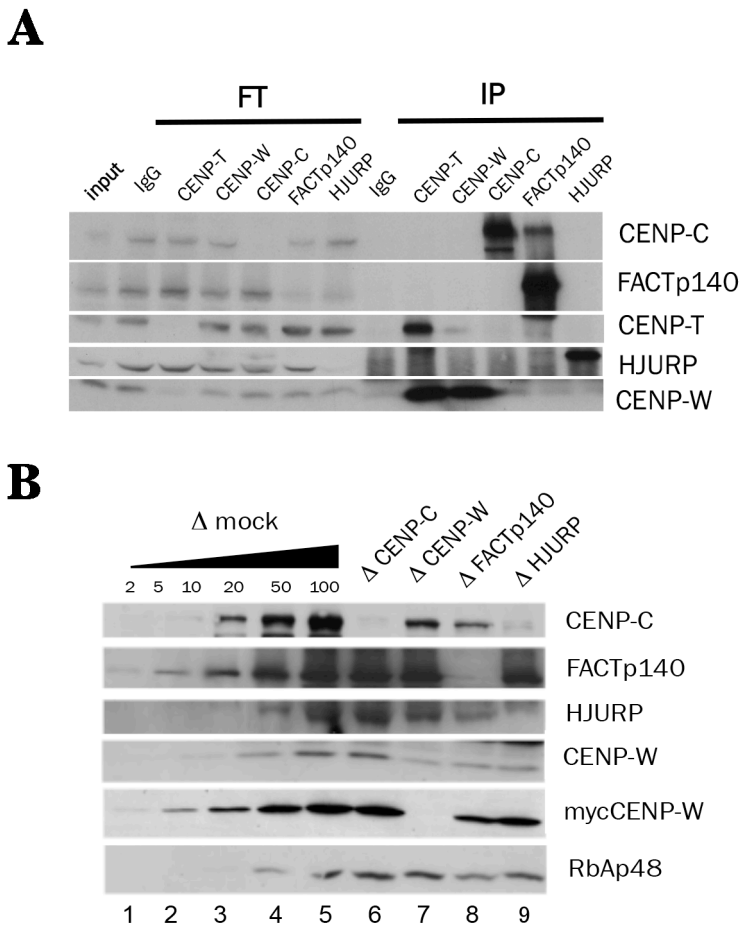


Figure R25. CENP-C is co-depleted by CENP-W, FACT and HJURP

A) Immunoblot analysis of immunoprecipitates (IP) obtained from CSF extracts with antibodies against CENP-T, CENP-W, CENP-C, FACTp140 and HJURP. As control, immunoprecipitation with non-immune rabbit IgG was used. Input extract was analyzed as well as the aliquots of flow-through (FT) of immunoprecipitations.

B) Egg extracts containing exogenously added mycCENP-W were depleted from CENP-C, CENP-W, FACT and HJURP. To check the extent of each depletion and co-depletion of the different proteins in every condition, 1.5ul aliquots were analyzed by immunoblot along with the ladder of mock-depleted extract (expressed as percentage of a 1.5-μl aliquot). RbAp48 is used as a loading control.

5.2 Role chromatin remodeler FACT in centromere propagation and function in *Xenopus* egg extracts

5.2.1 FACT complex localizes to centromeres in mitosis

To see if depleting FACTp140 subunit depletes the whole FACT complex, we have depleted the CSF extract from FACTp140 and checked how it affects FACTp87. The levels of FACTp87 in FACTp140 depleted extract were significantly reduced compared to the mock-depleted extract confirming that by depleting FACTp140 we are removing the whole complex from the extract (Figure R27A). Therefore, we could be sure that the observations obtained by using only the FACTp140 antibody are true for the whole FACT complex.

By immunofluorescence we found that FACTp140 localized to centromeres in CSF assembled chromosomes, although the signals were not very strong. In interphase nuclei the staining was spread all over the chromatin, consistent with an essential role of FACT in DNA replication previously reported. After entry into mitosis, FACTp140 accumulates again at centromeres (Figure R27B). As mentioned above, depletion of CENP-C from the soluble extract prevents this association of FACT with mitotic centromeres (Figure R26).

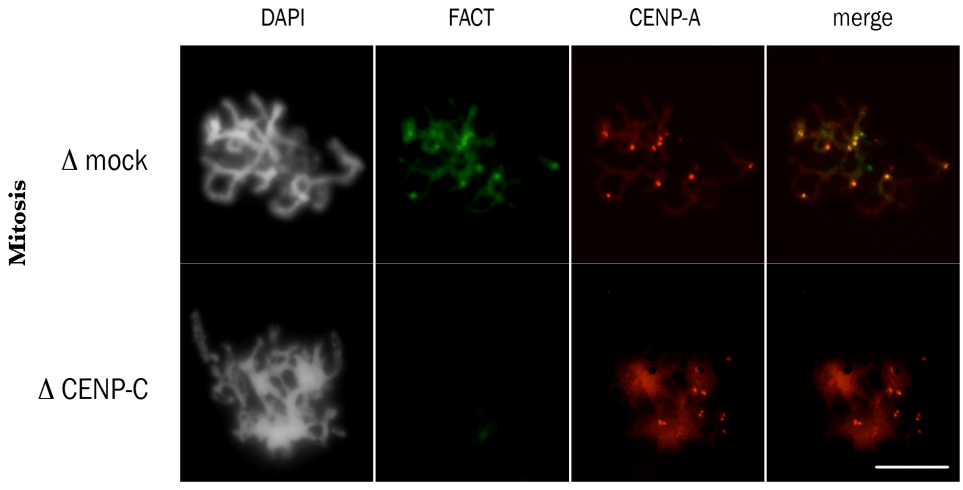


Figure R26. CENP-C is necessary for proper targeting of FACT to centromeres in mitosis.

Mitotic chromosomes were assembled in a mock depleted extract and an extract depleted from CENP-C, fixed and stained for FACTp140 (green) and CENP-A (red). DNA was counterstained with DAPI (white). Scale bar is 10 μm.

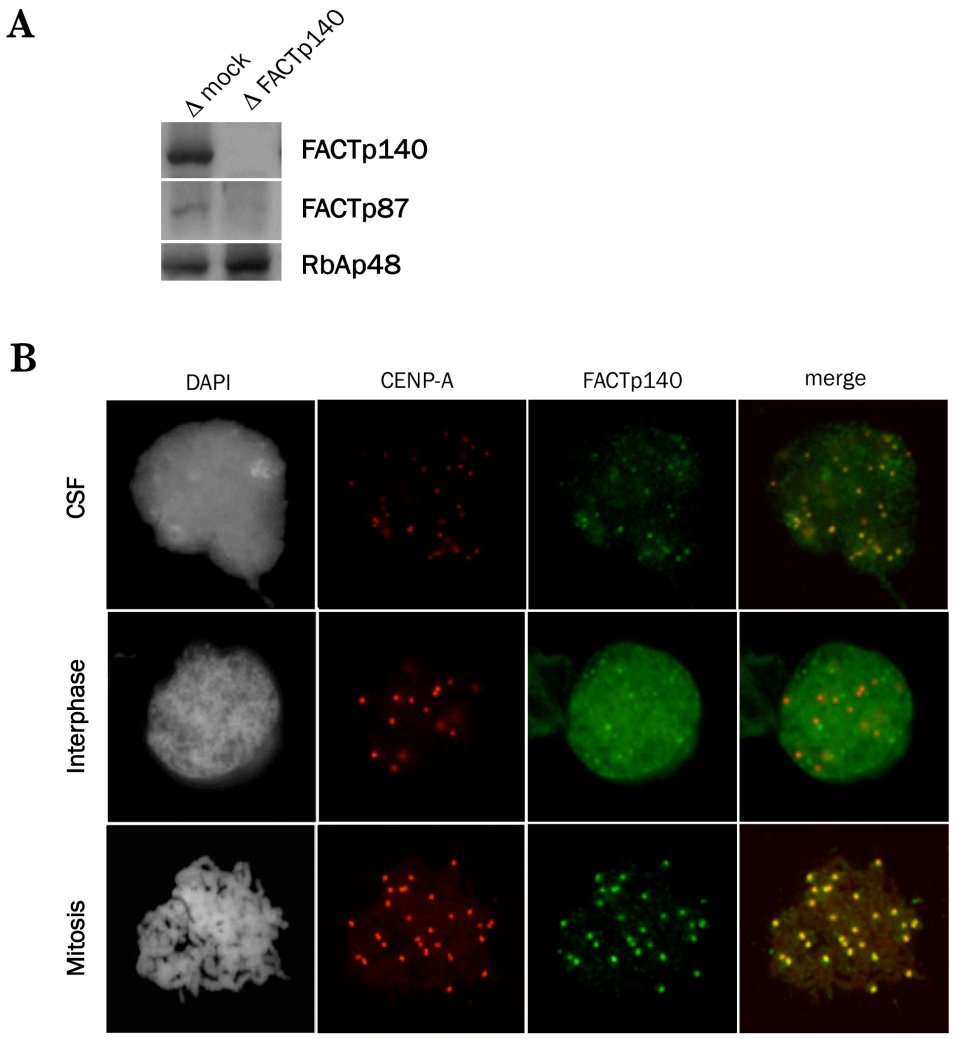


Figure R27. Centromeric localization of FACTp140.

A) Immunoblot analysis of mock-depleted and FACTp140 depleted CSF extract (Δ mock and ΔFACTp140). RbAp48 serves as loading control.
B) Chromosomes assembled in CSF, interphase and mitosis were stained with antibodies recognizing FACTp140 (green) and CENP-A (red) and counterstained with DAPI (white).

5.2.2 FACT is essential for the CENP-A deposition

We next asked if FACT has a role in CENP-A assembly. Since FACT depletion largely co-depletes CENP-C (Figure R28A), we expected that chromosomes assembled in FACT depleted extracts would have a severe defect in CENP-A deposition. This was indeed the case (Figure R28B). Addition of *myc*CENP-C mRNA to the FACT-depleted extract restored CENP-C levels both in the soluble extract (Figure R28A, lane 8) and at the centromeres of CSF chromosomes (Figure R28C). However, we did not observe a rescue of CENP-A deposition under this condition (Figure R28B). These data strongly suggest that FACT has an essential a role in CENP-A assembly that is independent of CENP-C.

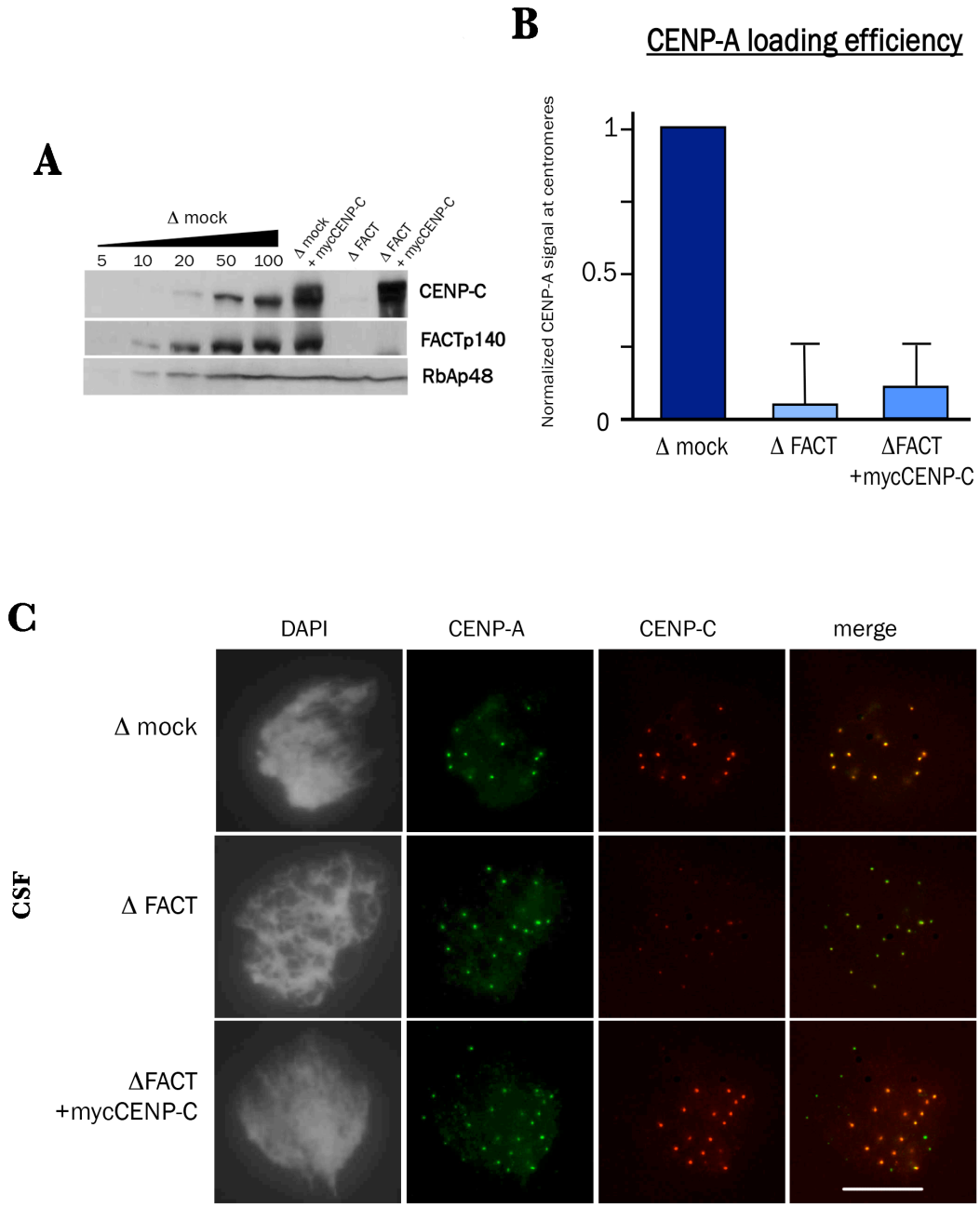


Figure R28. FACT has a role in CENP-A loading.

A) Immunoblot analysis of increasing amounts of a mock-depleted CSF extract (Δ mock, expressed as percentage of a 1.5-µl aliquot) and 1.5-µl aliquots of FACT depleted CSF extracts (Δ FACT) without or with added mycCENP-C mRNA. RbAp48 serves as loading control.

B) Bar graph representing CENP-A loading efficiencies in nuclei assembled in the indicated extracts. More than 250 centromeres were quantitated per condition and experiment. Error bars, SEM. n≥5

C) CSF chromosomes assembled in extracts described in A) were fixed and stained for CENP-A (green), CENP-C (red) and DNA (DAPI white) to confirm CENP-C targeting to centromeres when its levels are restored in the FACT depleted extract. Scale bar10 µm.

5.2.3 FACT is not required for maintenance of CENP-A at centromeres

In human cells, the RSF remodeling complex has been proposed to be important for maintenance of CENP-A nucleosomes. Our previous results suggest that eviction of CENP-A nucleosomes takes places in chromatin assembled in the egg extracts and is prevented by, at least, condensin II (Bernad et al., 2011). We therefore asked whether the defect observed in CENP-A loading could be due to increased eviction rates in the absence of FACT. To answer this question we measured CENP-A intensity in chromosomes assembled in CSF extracts either mock depleted or depleted of FACT, using interphase chromosomes as reference. As control we also performed this assay in a condensin II depleted extract (Figure R29A). Depletion of FACT did not have an effect on the stability of CENP-A nucleosomes present on CSF chromosomes when compared to control extracts (Figure R29B). In contrast, CSF chromosomes lacking condensin II had lower levels of CENP-A. We conclude that FACT is not required for stabilizing CENP-A nucleosomes at centromeres in *Xenopus* egg extracts.

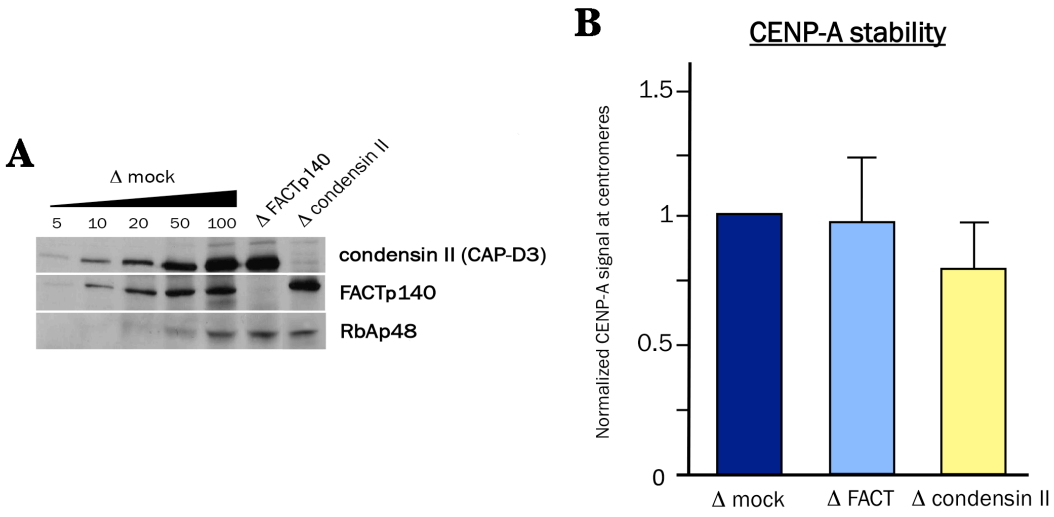


Figure R29. Defective CENP-A loading upon FACT depletion is not due to nucleosome eviction.

A) Immunoblot analysis of increasing amounts of a mock-depleted CSF extract (Δ mock, expressed as percentage of a 1.5-µl aliquot) and 1.5-µls aliquot of a FACT depleted (Δ FACT) and condensin II depleted (Δ condensin II) CSF extract. CAPD3 is a sbunit of the condensin II complex. RbAp48 is used as a loading control.

B) Quantification of CENP-A signal intensities in chromosomes assembled in CSF extracts mock depleted or depleted of FACT or condensin II in comparison with nuclei assembled in undepleted interphase extract. More than 250 centromeres were quantitated per condition and experiment. Error bars, SEM. n=3

5.2.4 Reduced incorporation of CENP-A at centromeres in the absence of FACT is not the consequence of aberrant incorporation outside centromeres

To confirm that the effect of FACT depletion on CENP-A is specific, we analyzed the amount of histone H3 on chromatin assembled in mock and FACT-depleted extracts by immunoblot. We did not detect a decrease in histone H3 but, if anything, a slight increase (Figure R30). This could be a consequence of the impaired loading of new CENP-A nucleosomes at centromeres upon FACT depletion that would normally reduce the levels of H3 nucleosomes at centromeric chromatin.

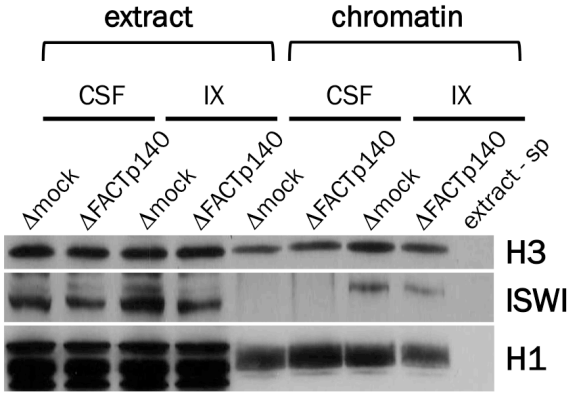


Figure R30. Defect in CENP-A loading upon FACT depletion is not due to a general reduction in nucleosome occupancy.

Immunoblot analysis of chromatin assembled in CSF, interphase (I) and mitotic (M) extracts and purified by centrifugation through a sucrose cushion (see Materials and Methods). As control, chromatin purified in the same way from a mock assembly reaction without sperm was used. Aliquots of the extracts were also analyzed. *Xenopus* ISWI is used as a marker for interphase, while histone H1 is used as a loading control.

In fission yeast, mutations in the histone chaperone FACT impair the maintenance of H3 chromatin on transcribed regions and promote widespread CENP-A incorporation at non-centromeric sites (Choi et al., 2012). Although not much transcription occurs in the *Xenopus* egg extracts, we checked whether we could observe an increase in ectopic incorporation of CENP-A in the absence of FACT. For that, we measured CENP-A staining outside centromeres in the same interphase nuclei used for assessing centromeric CENP-A deposition in mock and FACT depleted extracts. No significant differences could be detected (Figure R31). Taken together, we conclude that it is unlikely that the effect of FACT depletion on CENP-A loading at centromeres is due to its aberrant deposition at non-centromeric locations or to a general eviction of canonical and non-canonical nucleosomes.

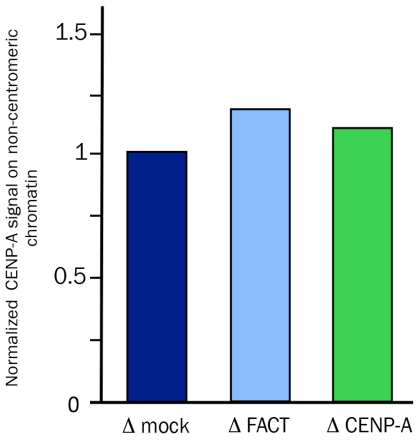


Figure R31. Defect in CENP-A loading upon FACT depletion is not due to aberrant deposition at non-centromeric locations.

Quantification of CENP-A fluorescence intensity at non-centromeric interphase chromatin assembled in mock-depleted and extracts depleted from FACT. We also analyzed interphase nuclei from CENP-A depleted extracts to have an idea of the background staining levels produced by the CENP-A antibody. Samples were normalized to the CSF control (mock depleted) sample. Integrated density was quantified for >75 non-centromeric chromatin areas (5 per nuclei, 15 nuclei) per condition.

5.2.5 A role for centromeric transcription in CENP-A deposition?

A recent report shows that RNA polymerase II-driven transcription of centromeric satellite sequences occurs in mitosis in human and mouse cells (Chan et al., 2012). Moreover, these centromeric transcripts promote recruitment/maintenance of CENP-C to centromeres (Wong et al., 2007). Experiments in using human artificial chromosomes also indicate that too little or too much transcription of centromeric satellites is detrimental for CENP-A deposition (Bergmann 2011, 2012). Since FACT is a cofactor of RNA pol II, one could envision that its role in CENP-A assembly could be related to centromeric transcription. To explore this possibility, we first tested the effect of treating the extract with ribonuclease (RNase) on CENP-A loading. We added two different concentrations of Ribonuclease A (RNase A), which cleaves single stranded RNAs, and incubated the extract for 1 or 2 hours at 22ºC before adding sperm chromatin. Aliquots of RNA preparations from the corresponding extracts were analyzed on an agarose gel to determine the efficiency of the RNase treatment (R32A). In both conditions, the levels of new CENP-A loaded onto chromatin where similar to an untreated extract (Figure R32B). We reasoned that maybe active transcription and not the transcripts *per se* are required for proper loading. In fact, unlike results in human cells, we found that CENP-C is properly targeted to centromeres in the extracts treated with RNAse (Figure R32C).

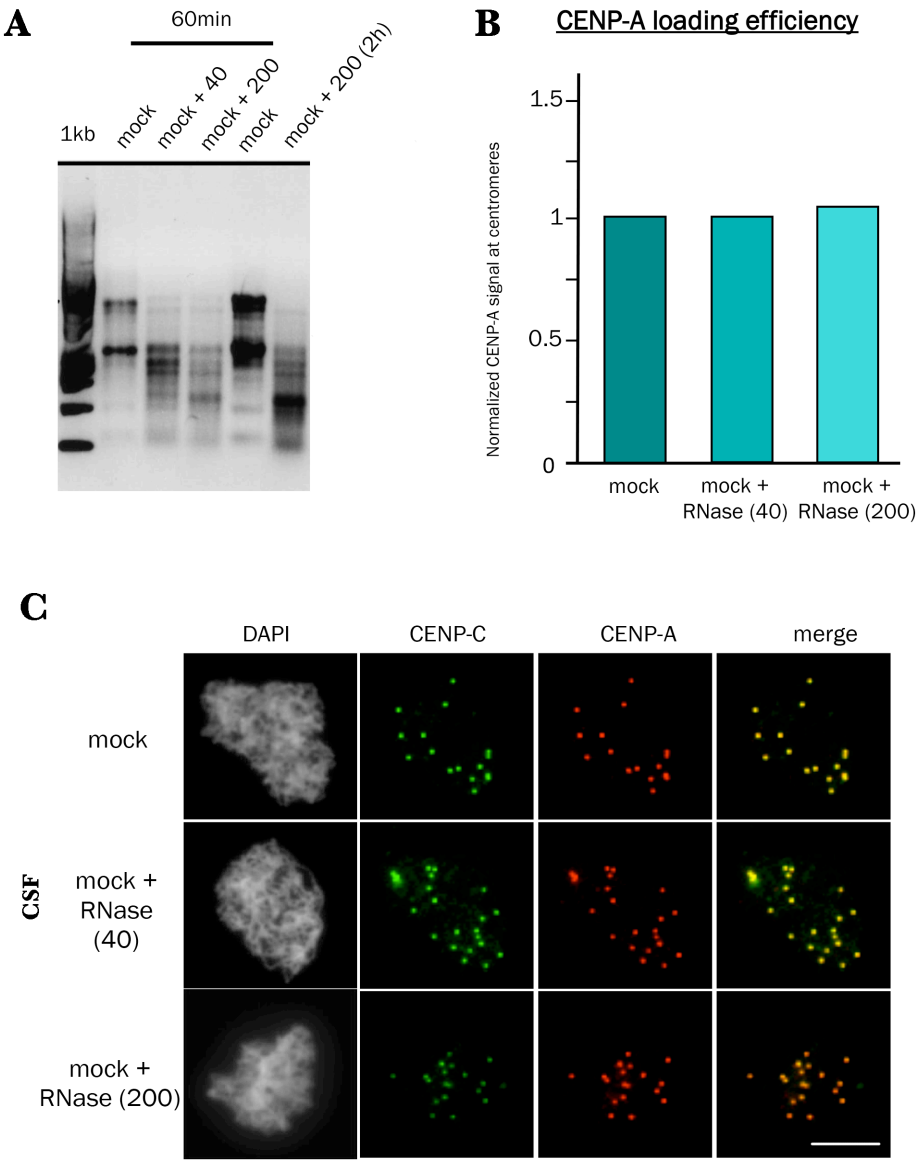


Figure R33 . Role of centromeric RNA transcripts in CENP-A assembly

A) Analysis of RNA samples prepared from the CSF extracts treated with increasing amounts of RNase A on agarose gel (40 and 200µl/ml). Conditions are indicated above the panel. As a size marker 1kb-ladder DNA marker was used. *Xenopus laevis* rRNA bands correspond to 4 kb and 1.8 kb.

B) Bar graph representing CENP-A loading efficiency in nuclei assembled in the indicated extracts. More than 250 centromeres were quantitated per condition and experiment. Error bars, SEM. n=3

C) Chromosomes assembled in CSF egg extracts after treatment with RNAse A were fixed and stained for CENP-A (red) and CENP-C (green). DNA was counterstained wit DAPI (white). Scale bar is 10 µm.

Next, we performed a CENP-A assembly assay upon RNA pol II inhibition with either α-amanitin or actinomycin D. The former is a peptide that binds to RNA pol II and inhibits its translocation on DNA whereas actinomycin D binds to DNA at the transcription initiation complex and prevents elongation. CENP-A loading was significantly impaired when the extract was treated with actinomycin D whereas increased loading was observed in the a-amanitin treated extract (Figure R33A). At least for actinomycin D, we observe partial inhibition of DNA replication at the lower concentration tested (2µl/ml). Centromeric localization of CENP-C in CSF chromosomes is disturbed in this condition, which could be partly responsible for the CENP-A loading defect (Figure R33B). Thus, our preliminary results are consistent with a role of centromeric transcription for CENP-A assembly also in *Xenopus* egg extracts. It will be necessary to explore further the appropriate drug concentration in these assays in order to avoid unspecific effects. Moreover, we will need to find a way to check effective RNA polymerase inhibition and set up a protocol to isolate small RNAs from the egg extracts.

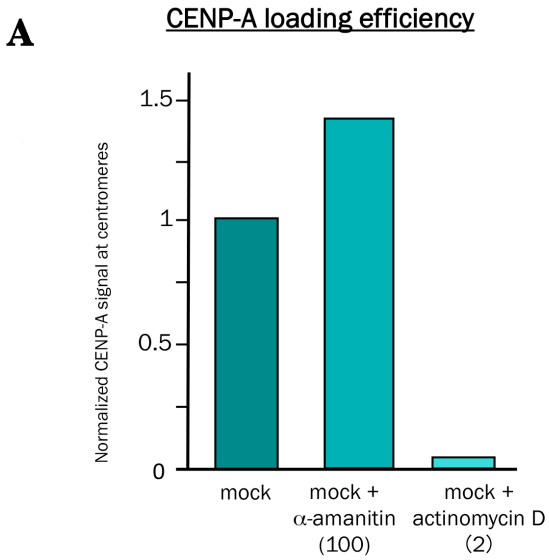


Figure R33. Inhibition of RNA pol II by actinomycin D abolishes the loading of CENP-A and affects the localization of CENP-C to centromeres

A) Quantification of CENP-A fluorescence intensity at centromeres for the assembly reaction in extracts treated with different inhibitors of RNA polymerase II (100µl/ml of α-amanitin and 2µl/ml of actinomycin D). Samples were normalized to CSF control sample. Integrated density per centromere was quantified for >250 centromeres. Error bars, SEM. n=2

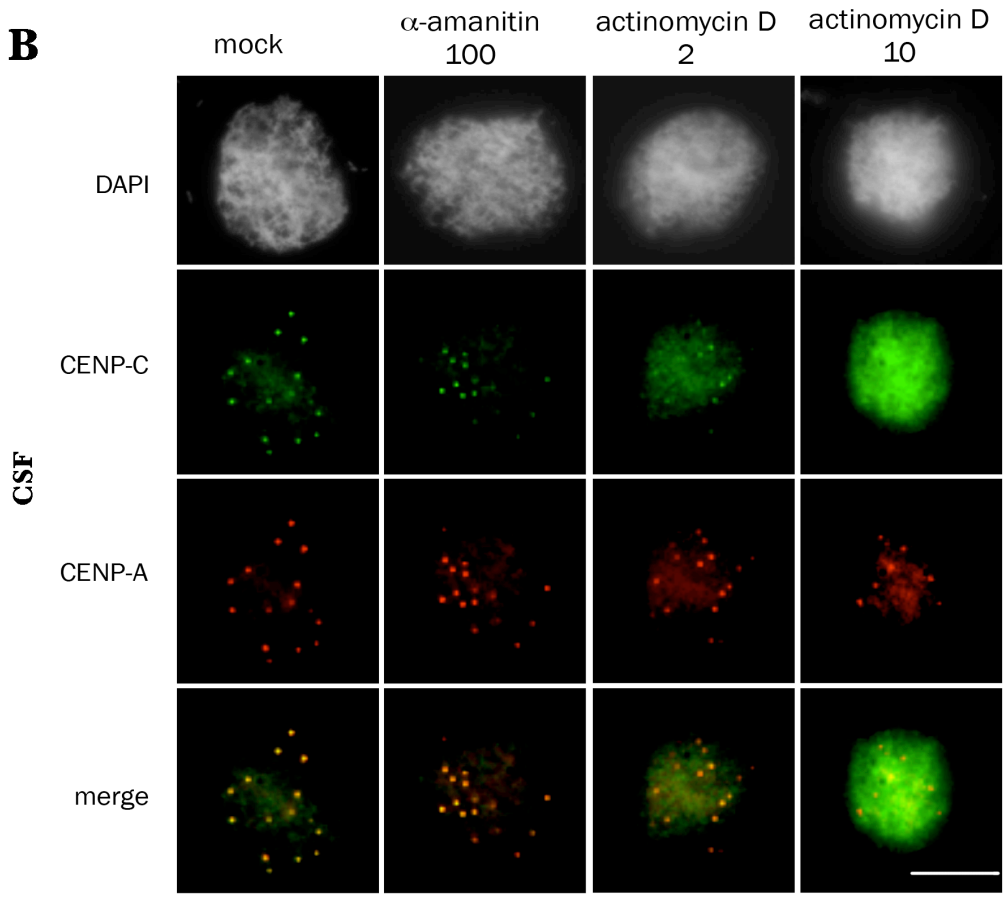


Figure R33. Inhibition of RNA pol II by actinomycin D abolishes the loading of CENP-A and affects the localization of CENP-C to centromeres (continued).

B) Chromosomes assembled in CSF extracts treated as described in A) were fixed and stained for CENP-C (green), CENP-A (red) and with DAPI (white). Scale bar is 10 μm.

Discussion

6. Discussion

The main objective of this thesis was the characterization of *Xenopus laevis* Constitutive Centromeric Associated Network (CCAN) proteins CENP-C, CENP-T and CENP-W. Functional examination of the role of these proteins in vertebrate cells reveals a complex network of interdependencies and, in some aspects, unclear results. We expected that the use of more simple system, the *Xenopus* egg cell-free extracts, would allow us to clarify some of these results, including the requirements of their association with chromatin and their roles in CENP-A deposition and kinetochore assembly.

6.1 Stepwise assembly of CENP-C, CENP-T and CENP-W

One of the defining futures of human CCAN proteins is that they are present at all times at centromeres (hence the name "constitutive"). However, as centromeres duplicate, new proteins have to be deposited on chromatin. Quantitative microscopy has also shown that CCAN proteins display different association dynamics throughout the cell cycle (Hemmerich 2008; Hellwig 2008; Hellwig 2009; Pendergast 2011; Eskat 2012). Since sperm chromatin added to the egg extract only contains CENP-A, this is also a good experimental system to explore the stepwise assembly of kinetochore proteins.

We observed that, although belonging to the same protein complex closely associated to centromeric nucleosomes (NAC), CENP-C, CENP-T and CENP-W do not associate at equal times to centromeres (Figure D1). Upon addition of sperm chromatin to the CSF extract, CENP-C is recruited to the centromeres (this study; Milks 2009). Neither CENP-T nor CENP-W can be detected at centromeres at this time and, consistently, CENP-C recruitment does not depend on CENP-T/W. Increasing evidence suggest that this recruitment relies on direct recognition of CENP-A by CENP-C (Carroll 2010, Kato 2013). Interestingly, when new CENP-A is deposited on the sperm chromatin template in interphase, we can measure a similar increase of CENP-C.

CENP-T is recruited to centromeres after the extract is released into interphase. In human cells, the amount of CENP-T/W almost doubles in late S phase (Pendergast 2011). Since CENP-T/W binds H3 nucleosomes present in centromeric chromatin (Hori 2008), it has been suggested that incorporation of H3 in CENP-A-containing chromatin during DNA replication provides new binding sites for CENP-T/W (Pendergast 2011). In our system, CENP-T binding is independent of DNA replication. It is likely that sperm chromatin, even in its unreplicated form, presents a configuration of alternating CENP-A and H3 nucleosomes that allows CENP-T recruitment.

It is unclear how CENP-T recognizes centromeric chromatin. We have shown that CENP-C is not strictly necessary for this recognition although its presence appears to stabilize CENP-T binding, particularly in mitosis. Our data also indicate that CENP-T recruitment requires some cell cycle-regulated mechanism since the CENP-A/H3 configuration of centromeric chromatin is the same in sperm chromatin in CSF and interphase extracts containing aphidicolin, but CENP-T binds to centromeres only in interphase.

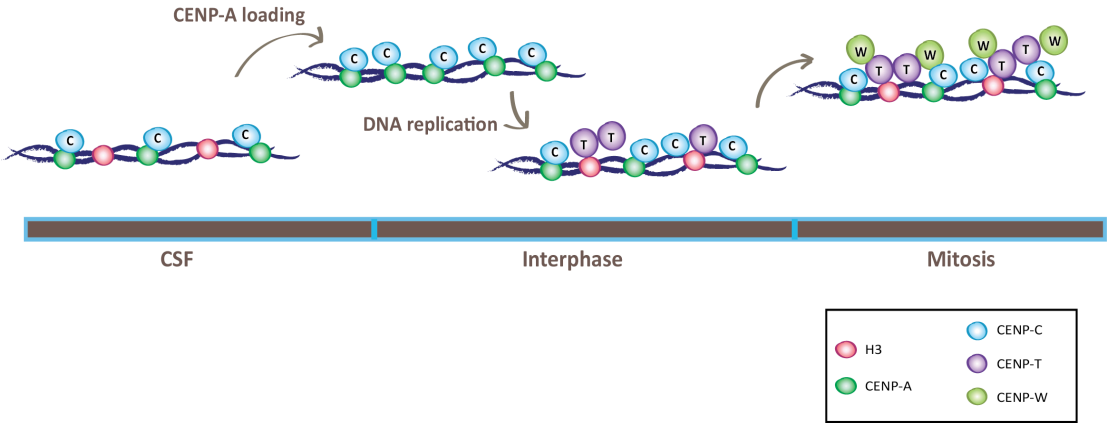


Figure D1. Stepwise assembly of CCAN proteins at centromeres

CENP-C recognizes CENP-A nucleosomes and is targeted to the centromere already in CSF. Upon release into interphase new CENP-A is loaded generating new CENP-C binding sites. Also in interphase CENP-T recognizes H3 nucleosomes in the vicinity of CENP-A nucleosomes by some unknown mechanism that does not depend on the presence of CENP-C. CENP-W is the last to come to centromeric chromatin. It does so in mitosis, probably binding to the CENP-T that is already there. CENP-C stabilizes the binding of the both CENP-T/W.

Unexpectedly, CENP-W does not associate with centromeres in interphase but once the extract is driven back to mitosis. This is at odds with the fact that CENP-T and CENP-W form a complex in the soluble *Xenopus* egg extract, as they do in chicken DT40 and human cells (Hori et al 2008; Pendergast et al, 2011). There is a fraction of CENP-T in the soluble egg extract that is not a part of CENP-T/CENP-W complex (Figure R1). This fraction could be recruited to centromeres in interphase and only later on in mitosis, the CENP-T/CENP-W complex would be recruited by recognizing CENP-T already present there. Indeed, the higher levels of CENP-T in mitosis compared to interphase are visible in chromosome assembly immunoblot (Figure R11) suggesting that the increase in CENP-T could be due to CENP-T/CENP-W recruitment to centromeres. This hypothesis can also explain that CENP-W depletion does not prevent CENP-T targeting whereas CENP-T depletion abolishes CENP-W targeting. It has been suggested from in vitro and crystallographic data

that CENP-T, -W, -S and –X, all four CCAN components containing histone-fold domains, form a heterotetramer that binds and bends DNA (around 100 bp) similar to canonical histones to form a nucleosome like structure (Nishino 2012). Whether this putative nucleosome-like complex exists throughout the cell cycle is unclear. In Fluorescence Recovery After Photobleaching (FRAP) experiments in human cells, CENP-T and CENP-W showed kinetic differences in late S-G2 cells that remain to be explained in molecular detail. However, no recovery is observed in G1 or early S phase, suggesting that after DNA replication and mitosis a switch takes place that stabilizes CENP-T/W at centromeric chromatin (Pendergast 2011). We speculate that a similar switch occurs in mitosis in *Xenopus* upon recruitment of CENP-W (and possibly the orthologues of CENP-S and CENP-X, which are also encoded in the *Xenopus* genome). Finally, the possibility cannot be excluded that CENP-T has a function in interphase independent of CENP-W. In fact, mutations in the yeast ortholog of CENP-T, Cnn1, have been reported to affect S phase progression (Bock 2012).

In summary, CENP-C is necessary for proper localization of CENP-T/W in mitosis, although a fraction of CENP-T can bind interphase centromeres lacking CENP-C. This suggests that either CENP-C stabilizes CENP-T and CENP-W at centromeres directly, or maybe upon CENP-C depletion the whole centromeric region is disrupted and no other centromeric protein can be recruited to this site. Actually, knocking down CENP-C in human or chicken DT 40 cells prevents association of almost all other CCAN proteins, including CENP-N, CENP-H, CENP-I, CENP-K and CENP-T (Carroll et al, 2010; Gascoigne et al, 2011) further confirming the importance of CENP-C as an essential centromere associated protein.

One intriguing observation is that CENP-C interacts with CENP-W in the soluble egg extract. Although these two proteins are often found next to each other in drawings depicting the inner kinetochore, a physical interaction between them in mammalian cells has not been reported. Thus, this could be a species-specific interaction. CENP-W is a very small and acidic protein (pI 10.5). *Xenopus* and human CENP-C proteins are highly homologous in their C terminal halves where most functional domains have been mapped to date, but differ considerably in their N terminus. We are currently preparing a set of CENP-C deletion constructs in order to map the CENP-C domain required for the association with CENP-W. The direct interaction between CENP-W and CENP-C may well serve to stabilize the inner kinetochore. The importance of having stored in the egg cytoplasm a fraction of CENP-W associated with CENP-C is unclear, since CENP-W cannot be found on chromatin until mitosis, when both CENP-C and CENP-T are already present.

6.2 CENP-C is essential for CENP-A deposition

Using our CENP-A loading assay, we demonstrate that CENP-C is essential for the proper assembly of CENP-A to centromeres in *Xenopus* egg extract whereas CENP-T and CENP-W are not. Previous studies have shown that CENP-C is required for CENP-A assembly also in *Drosophila* (Erhardt et al, 2008). The role of CENP-C in the *de novo* deposition of CENP-A in mammalian cells was unclear (Kwon 2007: Gascoigne 2011), which is not surprising given the key role of CENP-C in kinetochore assembly. In our in vitro system we are looking at the first round of CENP-A loading and the problems in chromosome segregation due to kinetochore defects are not important.

How might CENP-C contribute to CENP-A deposition? We here describe that CENP-C interacts with several factors implicated in this process in the soluble egg extract (Figure D2). One of them is M18BP1, a component of the Mis18 complex (Fujita 2007). Current evidence suggests that this complex recruits the CENP-A chaperone HJURP to centromeres (Barnhart et al, 2011) although it may also help priming centromeric chromatin for the exchange of H3 and CENP-A nucleosomes by recruitment of histone modifiers and the DNA methyltransferase 3A/B (Kim et al, 2012; Dambacher et al, 2012; Gopalakrishnan et al, 2009). Our own antibodies against M18BP1 do not work for immunofluorescence. However, consistent with the interaction that we observe in CSF extracts between CENP-C and M18BP1, Moree et al (2011) were able to show that depletion of CENP-C impairs M18BP1 localization to centromeres in CSF chromosomes and that immunodepletion of M18BP1 reduces CENP-A assembly. Taken all their results together with the immunoprecipitation data shown in Figure R19, it is likely that CENP-C brings M18BP1 to centromeres already in CSF, M18BP1recruits Mis18 in early interphase and Mis18 is the one that brings HJURP. Another conclusion from our data is that the so-called Mis18 complex is not such so that the absence of M18BP1 and Mis18 is not necessarily synonymous.

At odds with the cascade just described, we have also found that CENP-C associates with HJURP. If this interaction brings HJURP to centromeres, then the targeting function of M18BP1 should be irrelevant; at least as long as the maternal load of CENP-C lasts. In their experiments, Moree et al (2011) measure assembly of exogenously added mycCENP-A, not endogenous CENP-A, and this assembly requires also the addition of exogenous HJURP to the extract. Under these conditions, the targeting function of M18BP1 is likely to be required since there would be little free CENP-C in the extract ready to bind and guide the exogenous HJURP.

Finally, CENP-C associates also with FACT and our results reveal that this remodeling complex is required for CENP-A deposition (further discussed in the section 6.4). Since depletion of CENP-C prevents recruitment of FACT to centromeres, this could also explain the failure of the CENP-A assembly process when there is no CENP-C in the extract.

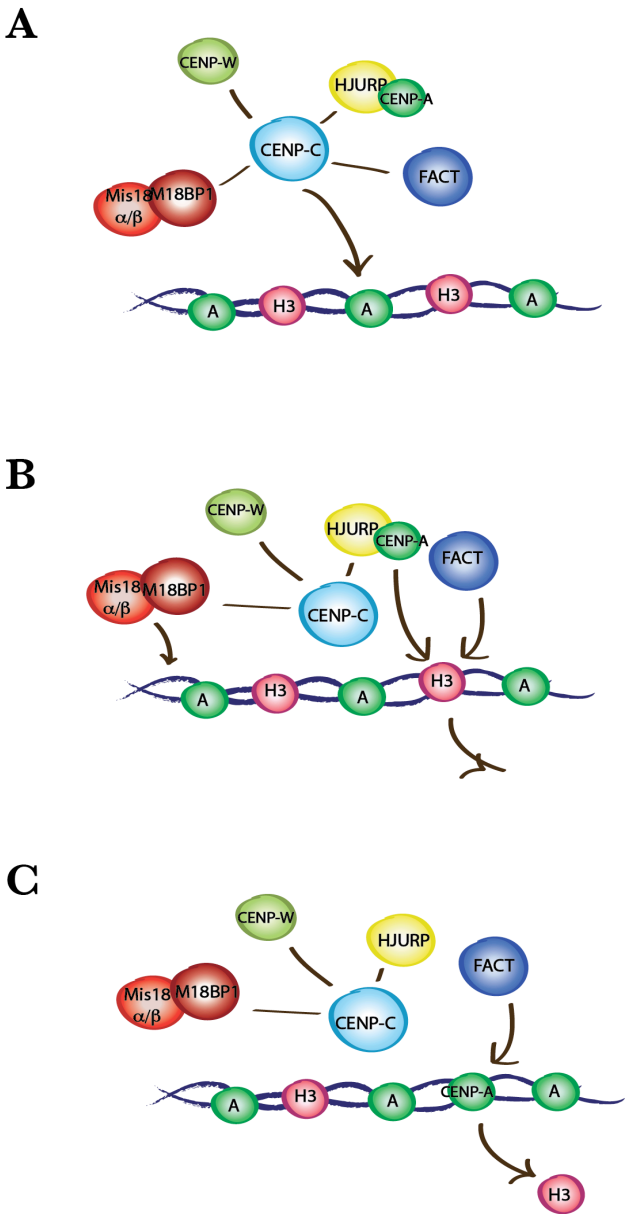


Figure D2. A model for CENP-C action in *Xenopus* extracts.

CENP-C brings M18BP1, CENP-W, HJURP and FACT to centromeres by recognizing CENP-A nucleosomes on chromatin (A). Mis18 complex (Mis18/M18BP1) probably modifies chromatin to be permissive for CENP-A deposition. HJURP brings CENP-A and together with FACT assembles new CENP-A nucleosome onto chromatin (B). In turn, H3 nucleosomes are discarded from centromeric chromatin (C).

6.3. CENP-C serves as a hub for centromere assembly in the early embryonic cycles

The cytoplasm of the frog oocyte is loaded with nutrients, structural components of the cell and preassembled protein complexes that will allow the zygote to carry out the first 11 cycles very quickly and with little transcription and protein synthesis. These cell cycles last around 30 min and consist essentially of a regular alternation of very rapid DNA replication and mitosis. Efficiency is achieved at the cost of fidelity. For example, there is no spindle assembly checkpoint in the egg extract. Our results clearly show that CENP-C interacts with CENP-W, M18BP1, HJURP and FACT in the soluble egg extract. With the exception of M18BP1 for which we cannot perform an efficient immunodepletion, we have observed that removal of any of the other abovementioned proteins largely removes CENP-C and affects the recovery of CENP-C in the immunoprecipitates of the others. This suggests that they form a large complex, although an alternative possibility is that separate complexes exist in equilibrium in the extracts so that removal of one of them affects the rest. As mentioned before for CENP-W, we plan to use CENP-C deletion constructs in order to map the CENP-C domains/motifs required for the distinct interactions described. What seems clear is that CENP-C might act as a protein hub that brings others for the process of centromere/kinetochore assembly. We speculate that the existence of these preassembled complex(es) in the egg will facilitate their recruitment to centromeres in the rapid embryonic cycles that follow fertilization.

6.4 The role of FACT in CENP-A deposition

Both subunits of FACT were found to co-purify with CENP-A in mammalian cells (Foltz et al., 2006; Obuse et al., 2004; Okada 2009). Knocking down FACT in chicken DT40 cells had a drastic effect in the incorporation of new CENP-A but it also stopped cell cycle progression (Okada 2009). Thus, the authors could not exclude the possibility that the observed defect was the consequence of cells not reaching the cell cycle window (early G1) in which CENP-A incorporation takes place. We bypass this problem in the *Xenopus* egg extracts. FACT depletion impairs DNA replication, but CENP-A loading does not depend on replication (Okuhara 1999; Bernad 2011). The results presented in this Thesis clearly indicate that FACT is required for CENP-A deposition in *Xenopus*. Moreover, FACT associates with CENP-C in the soluble extract and localizes to centromeres specifically in mitosis, just before deposition takes place, in a CENP-C dependent manner.

It was recently suggested that in *S. cerevisiae* FACT has a role in maintaining CENP-A at centromeres and preventing its aberrant assembly at non-centromeric sites (Choi et al, 2012). Using an eviction assay we showed that the CENP-A assembly defect observed in FACT depleted extracts is not due to problems in maintenance of CENP-A at centromeres. We also measured CENP-A staining in non-centromeric chromatin areas and did not observe an increase in FACT depleted samples. This suggests that FACT does not act in preventing CENP-A assembly all over chromatin.

It remains to be seen how FACT contributes to CENP-A assembly. As mentioned in the Introduction, HJURP is capable of assembling octameric CENP-A nucleosomes onto plasmid DNA in vitro (Barnhart et al., 2011 Shuaib et al., 2010) but it is likely that additional chromatin remodeling activities are required for the exchange of H3 nucleosomes in vivo. Considering its mechanism of action in replication and transcription, FACT could provide the same function in the assembly of CENP-A nucleosomes at centromeres. FACT interacts with both H2A-H2B and H3-H4 dimers but seems to preferentially remove H2A-H2B from DNA, and promote H3-H4 incorporation (Belotserkovskaya et al., 2003). FACT could therefore help H3 nucleosome disassembly to promote the subsequent incorporation of CENP-A-H4 by HJURP. In *Drosophila*, FACT was shown to facilitate replacement of canonical H3 nucleosomes with H3.3 nucleosomes (Nakayama et al., 2007). There are additional possibilities. In *S. cerevisiae*, the reassembly of chromatin on transcribed templates with recycled histones is defective in the *spt16* (FACTp140) mutant. Thus FACT normally prevents the incorporation of free histones by recycling pre-existing histones in template-associated nucleosomes during transcription (Jamai et al, 2009). In the context of the centromere, FACT might act to promote incorporation of CENP-A-H4 to centromeres in concert with HJURP or it might prevent the incorporation of free H3-H4 onto chromatin at sites where H3-H4 was removed to make place for CENP-A-H4 (Figure D3). It will be important to design and perform *in vitro* assays to measure nucleosome incorporation in centromeric DNA upon FACT addition. One could think of a histone exchange assay to measure the ability of HJURP either alone or together with FACT, to incorporate CENP-A in a chromatin template made of H3 nucleosomes {Mizuguchi et al, 2004}.

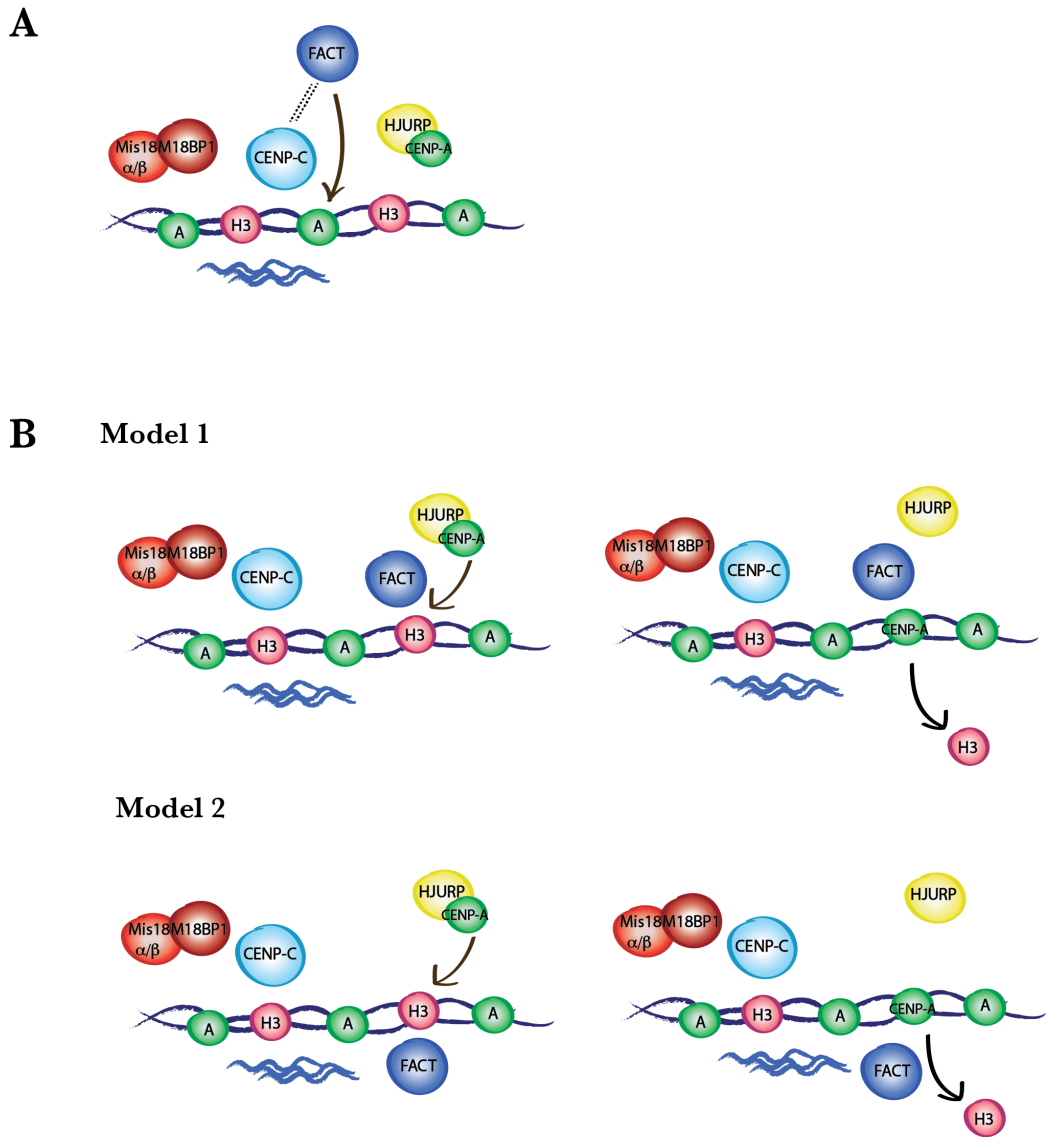


Figure D3. The role of FACT in CENP-A loading.

A) FACT localizes to centromeres in mitosis in a CENP-C dependent manner.
B) FACT might act by helping the assembly of CENP-A onto chromatin together with HJURP (Model 1) or by assuring the removal of H3 nucleosomes in the places where CENP-A nucleosomes are going to be loaded (Model 2).

6.5 Centromeric transcription and CENP-A loading

Recent evidences support a role for transcription in centromere propagation and function. Tethering H3K4me2-specific demethylase LSD1 to the centromeric region of a human artificial chromosome (HAC) caused a decrease in centromeric transcription from the alphoid satellite DNA, failure to recruit HJURP and thereby a defect in the incorporation of

newly synthesized CENP-A (Bergmann et al, 2011). Tethering a potent transcriptional activator to induce high levels of alphoid transcription resulted in rapid inactivation of the HAC while also impairing the CENP-A loading (Bergmann et al, 2012). These results imply that centromeric transcription could have a role in CENP-A propagation and maintenance, but also suggest that in order to have a functional centromere, this centromeric transcription has to be tightly regulated.

Recent experiments in HeLa cells have also reported active transcription by RNA pol II at centromeres during mitosis (Chan et al, 2011). Transient inhibition of RNA pol II activity caused a decrease in centromeric transcripts, reduced CENP-C at centromeres and an increase in lagging chromosomes (Chan et al, 2011l). This is consistent with previous studies showing that localization of CENP-C and the CPC component INCENP to centromeres depends on the presence of single-stranded centromeric a-satellite transcripts (Wong et al, 2007). In mouse cell lines, the transcripts derived from the centromeric minor satellite were also found tightly associated with centromere chromatin and with the chromosomal passenger complex (CPC) (Bouzinba-Segard et al, 2006; Ferri et al, 2009).

RNA pol II transcription requires the action of chromatin remodelers and histone chaperones. Since FACT aids RNA pol II activity, it could as well act in RNA pol II transcriptional activity through condensed CENP-A chromatin to promote centromeric transcription. This transcription could contribute to CENP-A loading in two ways. First, centromeric RNA might act as a molecular scaffold for the recruitment and organization of key centromere proteins, like CENP-C. Second, transcription would maintain the CENP-A domain in an "open" chromatin state that would facilitate CENP-A nucleosome incorporation by HJURP.

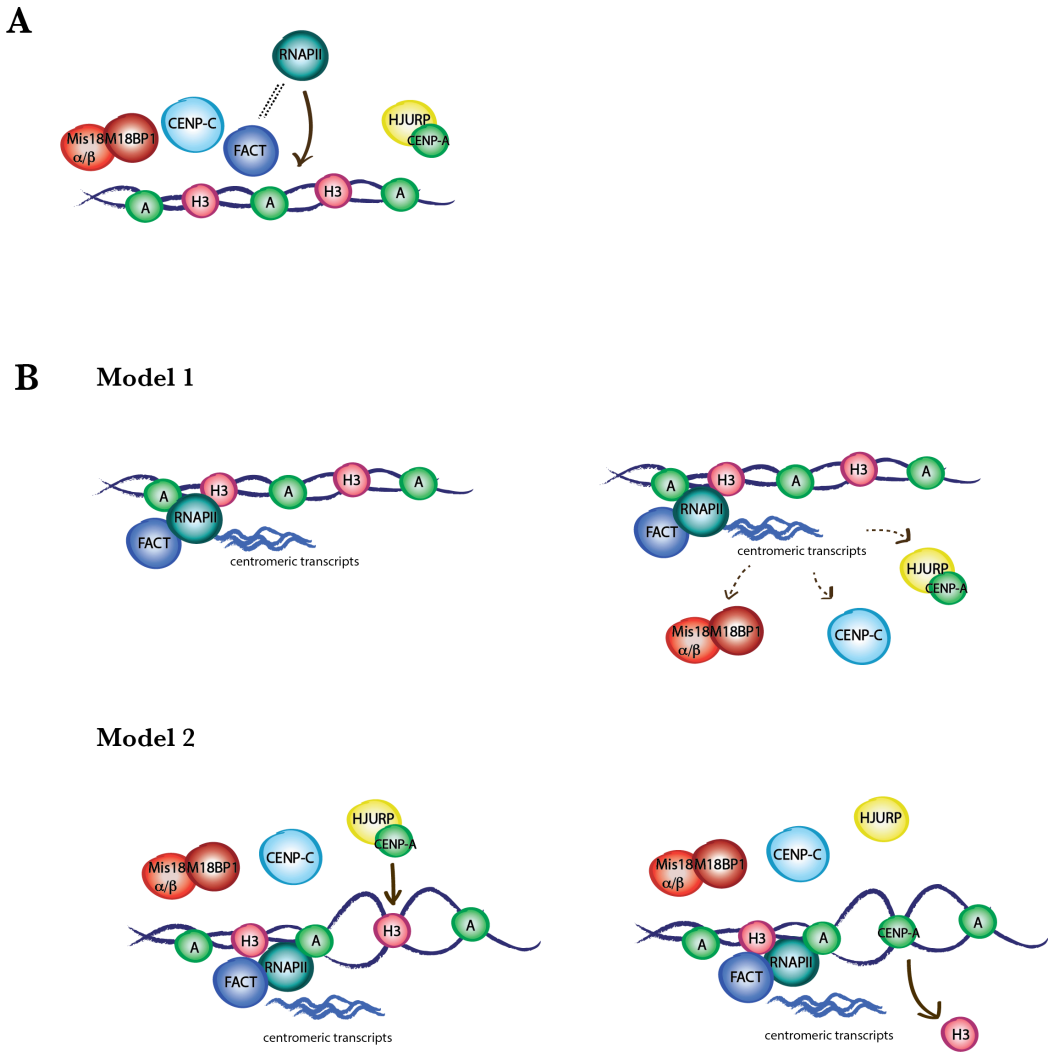


Figure D4. The role of transcription in CENP-A deposition.

A) FACT helps the activity of RNA polymerase II at centromeres.
B) RNA pol II produces centromeric transcripts, which help the recruitment of other centromeric proteins (Model 1) or facilitates the CENP-A deposition at centromeres by producing the open form of chromatin (Model 2).

Our preliminary results in *Xenopus* egg extracts indicate that treating the extract with RNAse does not prevent CENP-C targeting or CENP-A deposition. One caveat of these results is that we do not know if our treatment actually destroys the putative centromeric RNA transcripts. Among the sequences of ~300,000 unique small non-coding RNAs from *Xenopus* egg extracts, 3 aligned perfectly to the centromeric repeat (M.D. Blower, personal communication; Lau et al, 2009; Edwards and Murray, 2005). Thus, it is possible that centromeric satellites are indeed being transcribed in the egg, although at vey low rate. Increasing the ratio of chromatin to cytoplasm by addition of sperm chromatin to the egg extract we may be able to detect these transcripts by Northern blot. If so, this detection can be used as readout for finding appropriate conditions for inhibiting this transcription and

assessing the consequences for the incorporation of new CENP-A. Additional experiments to support a role of transcription in CENP-A deposition could include detection of RNA pol II at centromeres by immunofluorescence.

6.6 Centromeres and disease

The study of centromeres and the factors involved in their propagation and maintenance is key to understand the essential process of chromosome segregation. Aberrant chromosome segregation that leads to genomic instability and aneuploidy is the feature the majority of tumors. Moreover, centromeric proteins have been related to human diseases for some time. Auto-antibodies against CENP-A, CENP-B, and CENP-C were first detected in the sera of patients affected by scleroderma-spectrum disease (Moroi et al. *1980*; Earnshaw et al. *1987*). Many CENPs are associated with tumor promotion, including CENP-M, CENP-U/50 and CENP-W. Additionally, CENP-A and CENP-H were found to be overexpressed in colorectal cancers (Tomonaga et al, 2005, 2003). The CENP-A chaperone HJURP was recently proposed as a potential prognostic marker in treatment of breast cancer patients. Therefore, gaining knowledge on centromeres and the means of their propagation might help us understand better what occurs when these processes are disrupted and cause abnormalities and pathological phenotypes. Furthermore, we may then be able to design and target these proteins for better therapies in the treatment of diseases. Centromere proteins such as CENP-A and M18BP1 have no known role outside of mitosis, which makes them attractive targets for drug development. Inhibition of mitosis-specific mechanisms will diminish the possibility of undesirable side effects such as those seen with microtubule poisons like taxol.

6.7 Final remarks

Centromeres were first described by Walther Flemming (1882) as a primary constriction on mitotic chromosomes, where cellular fibers (the microtubules) attach. Many years later, electron microscopy images showed that a megadalton protein complex named the kinetochore assembles right on this primary constriction (Robbins and Gonatas, 1964; Brinkley and Stubblefield, 1966). A number of molecular players involved in centromere identity have identified over the years, particularly in the last decade. Despite extensive studies on the expression and localization of these centromere/kinetochore components throughout the cell cycle, we still lack a clear picture on how centromere specification occurs.

Several components of the outer kinetochore have homologues in almost all the eukaryotic organisms studied. For example, the KMN network that is essential for microtubule attachment at kinetochores is found in fungi, flies, worms and vertebrates (Lampert and Westermann, 2011). In contrast, non-mammalian homologues of the inner kinetochore - with the exception of CENP-C, which is widely conserved - have been more difficult to identify. Only recently a number of CCAN proteins including CENP-T and CENP-W have been reported in yeast (Bock et al, 2012, Schleiffer et al, 2012) whereas in *Drosophila melanogaster* and *Caenorhabditis elegans*, they are not found. Comparative understanding of the mechanism of centromere specification and function in different model organisms will allow us to deduce the general principles underlying this key process. Our studies in demonstrate that *Xenopus* egg extracts can faithfully recapitulate centromere propagation and kinetochore assembly providing us with the opportunity to dissect these processes in molecular detail in a cell-free system.

Conclusions

7. Conclusions

1. *Xenopus laevis* CENP-T and CENP-W form a complex in soluble egg extracts. A small fraction of CENP-W associates with CENP-C independently of CENP-T.
2. CENP-C, CENP-T and CENP-W are recruited to centromeres of chromosomes assembled in Xenopus egg cell free extracts at different times. CENP-C is recruited in CSF, CENP-T in interphase and CENP-W in mitosis.
3. CENP-C is necessary for proper localization of CENP-T/W in mitosis, although a fraction of CENP-T can bind interphase centromeres lacking CENP-C. Centromere localization of CENP-C does not depend on CENP-T or CENP-W.
4. CENP-C, CENP-T and CENP-W are all three essential for full kinetochore assembly in mitosis.
5. CENP-C is required for the loading of new CENP-A in interphase, while CENP-T and CENP-W are not.
6. In addition to CENP-W, CENP-C associates with the CENP-A chaperone HJURP and the chromatin remodeler FACT in the soluble egg extract.
7. FACT localizes to centromeres in mitosis in a CENP-C dependent manner and it is required for the loading of new CENP-A. The defect observed upon FACT depletion is not due to eviction of CENP-A nucleosomes or disruption of histones in general.

Conclusiones

Conclusiones

1. CENP-T and CENP-W aparecen formando un heterodímero en los extractos obtenidos de huevos de *Xenopus laevis*. Una pequeña fracción de CENP-W se asocia con CENP-C con independencia de CENP-T.
2. CENP-C, CENP-T y CENP-W se dirigen a los centrómeros de los cromosomas ensamblados *in vitro* en estos extractos en diferentes momentos. En tanto que CENP-C se detecta en los centrómeros de cromosomas obtenidos en extractos CSF, CENP-T aparece en los centrómero en interfase y CENP-W en la siguiente mitosis.
3. La localización centromérica de CENP-T y CENP-W en mitosis depende de CENP-C, si bien una fracción de CENP-T puede detectarse en interfase incluso en ausencia de CENP-C. La localización centromérica de CENP-C no require CENP-T ni CENP-W.
4. Tanto CENP-C como CENP-T como CENP-W son imprescindibles para un ensamblaje correcto del cinetocoro mitótico.
5. Sólo CENP-C juega un papel esencial en la incorporación de CENP-A en interfase. CENP-T y CENP-W no participan en este proceso.
6. CENP-C se almacena en el citoplasma de los oocitos asociado no sólo a CENP-W sino también a la chaperona de CENP-A HJURP y al complejo remodelador de cromatina FACT.
7. FACT se localiza en los centrómeros en mitosis de forma dependiente de CENP-C y su función es necesaria para la incorporación de nuevos nucleosomas CENP-A.

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